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# Interleukin-15 and [alpha](1,3)galactosyl-expressing vaccine combination therapy for melanoma

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**Interleukin-15 and  $\alpha(1,3)$ galactosyl-expressing vaccine combination therapy for melanoma**

by

**Dana Z Awwad**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:  
Charles Link, Co-major Professor  
N. Matthew Ellinwood, Co-major Professor  
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Iowa State University

Ames, Iowa

2007

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*To my mom and dad*

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## LIST OF ABBREVIATIONS

$\alpha$ Gal;  $\alpha(1,3)$ Galactosyl epitope

$\alpha$ GT;  $\alpha 1,3$ -galactosyltransferase enzyme

$\alpha$ GT KO;  $\alpha 1,3$ -galactosyltransferase knockout

APCs; antigen-presenting cells

B16 $\alpha$ Gal; B16F0 melanoma cells expressing  $\alpha$ Gal epitope

B16IL-15; B16F0 melanoma cells expressing IL-15

B16N/V; B16F0 melanoma cells

CFSE; 5,6-Carboxyfluorescein diacetate, succinimidyl ester dye

DCs; dendritic cells

GM-CSF; granulocyte macrophage colony-stimulating factor

IL-15; interleukin-15

i.v.; intravenous

NK; natural killer cells

RRBCs; rabbit red blood cells

s.c.; subcutaneous

TS; Tyrode's salt solution



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## ABSTRACT

The hyperacute immune response observed in humans after xenotransplantation is mainly induced by the  $\alpha(1,3)$ Galactosyl ( $\alpha$ Gal) epitopes expressed on xenografts and the pre-existing anti- $\alpha$ Gal antibodies that recognize these epitopes. Based on this hyperacute rejection, we hypothesized that human cancer cells genetically modified to express the  $\alpha$ Gal epitope could provide a new anti-cancer vaccine. This hypothesis was previously studied using the  $\alpha$ 1,3-galactosyltransferase knockout ( $\alpha$ GT KO) mouse model and B16 melanoma cells genetically modified to express  $\alpha$ Gal epitopes (B16 $\alpha$ Gal). In this model, the B16 $\alpha$ Gal vaccine showed efficacy in treating pre-existing subcutaneous and pulmonary  $\alpha$ Gal-negative B16 melanomas in mice. Furthermore, adoptively transferred lymphocytes from mice vaccinated with the B16 $\alpha$ Gal vaccine elicited a therapeutic response in mice bearing pulmonary tumors. In an attempt to increase the efficacy of the B16 $\alpha$ Gal vaccine, in the present study, IL-15-expressing vaccine cells (B16IL-15) were administered to mice in combination with the B16 $\alpha$ Gal vaccine with or without B16N/V vaccine cells (irradiated B16F0 melanoma cells). As documented in literature, IL-15 stimulates the proliferation and maintenance of memory CD8<sup>+</sup> T cells. We exploited these effects of IL-15 in preventive and therapeutic experiments, wherein B16 tumors were implanted either subcutaneously or intravenously in  $\alpha$ GT KO mice. In all the efficacy studies, the combination therapy showed efficacy in vaccinated mice versus control mice receiving no vaccination. In three out of five *in vivo* experiments, mice receiving the combination therapy were more effectively treated for B16 melanomas compared to mice receiving B16 $\alpha$ Gal vaccine (with or without B16N/V vaccine).

Moreover, long-term memory CD8<sup>+</sup> T cells adoptively transferred from mice receiving the combination therapy successfully treated mice with pulmonary B16 melanomas. *In vivo* 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling experiments demonstrated that mice receiving the combination therapy developed CD8<sup>+</sup> T cells which proliferated to a greater degree than CD8<sup>+</sup> T cells from mice vaccinated with B16 $\alpha$ Gal plus B16N/V vaccine. In conclusion, the results obtained in this study using the described murine system demonstrate a trend supporting the hypothesis that a combined therapy of B16 $\alpha$ Gal plus B16IL-15 vaccines provides a potential improved treatment outcome compared to the B16 $\alpha$ Gal vaccine alone.

## INTRODUCTION

### ***Cancer and Immunotherapy***

Cancer is a major health problem in developed countries including the United States where one in four deaths reported is due to cancer (1). Surgery, radiotherapy, and chemotherapy are the primary approved modes of treatment for cancer patients. However, the complications that accompany these therapies and the cases and forms of cancer refractory to these treatment modalities have prompted a search for more effective treatments with less harmful side effects (2). A promising therapy that has been extensively studied for the past two decades is active immunotherapy. Various cancer vaccines have already shown some degree of clinical benefit (3). The first cancer vaccines were composed of irradiated or inactivated whole tumor cells that were reported to express mutant antigens that rendered them more immunogenic (2). One potential advantage that active immunotherapy has over other therapies is specificity (2). Vaccines can target antigens that are only expressed by tumor cells, thereby rendering normal tissue unharmed. Our current efforts are focused on using whole tumor cells in order to provide a broader source of antigens. This may be advantageous since information is often lacking concerning the most relevant tumor antigens in most human cancers (3). Cancer vaccines can be designed to elicit both humoral and cellular immune responses. The source of cells for cancer vaccines can be either autologous or allogeneic tumor cells (4). Autologous vaccines have the advantage of containing patient specific and potentially rare antigens that are appropriately HLA-matched for optimum antigen

presentation to T cells. There are, however, two major disadvantages to autologous vaccines in treating human cancer: first, the impracticality of manufacturing this type of vaccine in a timely manner and in adequate quantities; and second, the great difficulty of cell characterization since the vaccines are customized for each patient. These important issues have skewed efforts at many institutions toward the production of well-characterized allogeneic tumor vaccines. Allogeneic vaccines could be made available in sufficiently large quantities to treat different patients and at all stages of the disease (4, 5).

### ***Whole Cell Cancer Vaccines***

Different approaches using cell-based vaccines are currently being explored in clinical trials. Some appear promising while others have been discontinued. For example, Canvaxin, developed by Dr Donald Morton in 1984 and used at the John Wayne Cancer Institute (JWCI) was an allogeneic whole-cell vaccine that consists of three melanoma cell lines selected from among more than 150 melanoma cell lines. This vaccine was designed to include more than 20 immunogenic melanoma-associated as well as non-melanoma tumor-associated antigens from these three cell lines. Canvaxin was intradermally injected as irradiated whole cells adjuvanted with *Bacillus Calmette-Guerin* (BCG), a strain of *Mycobacterium bovis* (6). The purpose of introducing an immune adjuvant is to further enhance the vaccine's immune response by recruiting antigen-presenting cells (APCs), macrophages, and B cells to the site of vaccination. In theory, MHC class II presentation by APCs and B cells were supposed to activate CD4<sup>+</sup> T cells which should further activate B cells and macrophages (7). Activated B cells were to

produce antibodies against tumor-specific antigens which might trigger complement destruction of tumor cells and antibody-dependent cellular cytotoxicity. Furthermore, through their Fc $\gamma$  receptors, APCs in turn would uptake tumor-specific antigens and present them to CD8<sup>+</sup> T cells, thus in theory eliciting a cytotoxic response. Patients vaccinated with Canvaxin showed humoral as well as cell-mediated responses against autologous melanoma-associated antigens (8). In 1998, phase III trials were initiated for the Canvaxin vaccine. Patients with stage III or IV melanoma were treated with Canvaxin vaccine with or without BCG after complete resection of metastatic melanoma. Unfortunately, in 2005, these trials were halted because of a lack of vaccine efficacy; vaccine treatment was no better than placebo treatment (4).

Another allogeneic whole-cell vaccine is GVAX which is composed of two irradiated prostate cancer cell lines, LN-CaP and PC-3, both genetically modified to produce granulocyte macrophage colony-stimulating factor (GM-CSF) (9). This cytokine acts as a chemo-attractant of granulocytes and differentiation factor that contributes to the maturation and activation of cells belonging to the myeloid lineage such as monocytes, neutrophils, macrophages, dendritic cells, and eosinophils (7). It participates in producing “danger signals” needed to activate the immune system, break tolerance, and stimulate an anti-tumor response (9-12). Importantly, when compared to multiple cytokines and growth factors transduced into B16 melanoma cell vaccines, GM-CSF was shown to be the most potent at eliciting an anti-tumor response (13, 14). Ten different B16 melanoma tumor cell vaccines were generated, each expressing a different cytokine or growth factor, and used to prevent a challenge with B16 melanomas in mice (13). Only irradiated B16 melanoma tumor cells genetically engineered to express GM-CSF conferred 100%

protection in mice. In addition, when the 10 cytokines were compared in the poorly immunogenic mouse tumor model, GM-CSF was classified as the most potent cytokine in terms of generating CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses. Additionally, vaccinated mice were not only able to survive both immediate and late challenge with B16 melanoma, but were also able to reject pre-established tumors. In multiple murine models, vaccination with irradiated cells expressing GM-CSF resulted in enhanced tumor antigen presentation by DCs and macrophages, enhanced CD4<sup>+</sup>, CD8<sup>+</sup>, and CD1d-restricted NK T cell responses, and enhanced antibody mediated protective immunity (15). Phase III clinical trials are currently under study for the GVAX vaccine.

For an optimal T cell activation to occur, T cells need to interact with a specific antigen (16) and engage with costimulatory ligands on APCs. Failure to bind to costimulatory ligands can render T cells anergic (17). The cytotoxic T lymphocyte antigen (CTLA-4) is a receptor on T cells that blocks the costimulatory signal by binding to B7 molecules on APCs. The administration of anti-CTLA-4 antibodies has enhanced anti-tumor responses in several animal tumor models (18, 19) and this antibody has been used in combination with prostate cancer vaccines (9). Recently, the National Cancer Institute has developed a vector-based vaccine that uses the same rationale of anti-CTLA-4 antibody (20). This vaccine is composed of vaccinia and fowlpox viruses expressing prostate-specific antigen (PSA) and three human costimulatory molecules, designated TRICOM (TRIad of COstimulatory Molecules: B7.1, intercellular adhesion molecule-1 (ICAM-1), and lymphocyte function-associated antigen-3 (LFA-3) (21)). The vaccine has shown clinical responses based on Response Evaluation Criteria in Solid Tumors

(RECIST) and drops in serum PSA in phase I/II trials in patients with advanced prostate cancer (3, 20).

Another immunotherapy cell-based vaccine is Sipuleucel-T (Provenge) which is composed of autologous dendritic cells (DCs) cultured with a fusion protein, PA2024, which consists of prostatic acid phosphatase (PAP), an antigen expressed in most prostate cancers but not by normal tissue, and GM-CSF (22-24). *Ex vivo* incubation with PA2024 will activate dendritic cells and upregulate their costimulatory molecules. These cells are then injected back into patients where they will hopefully home to the lymph nodes and activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells generating an effective antigen specific cellular immune response. Arguably, DCs are the most potent APCs and can solely initiate a T-cell immune response of naive T cells (25). Sipuleucel-T is now under study in a phase III clinical trial in hormone refractory prostate cancer (HRPC) patients.

### ***The $\alpha$ Galactosyl Epitope***

Based upon the hyperacute rejection found to occur with xenotransplants in primates, our laboratory developed a whole-cell Gal $\alpha$ 1,3Gal epitope ( $\alpha$ Gal)-expressing vaccine (HyperAcute vaccine). The  $\alpha$ Gal epitope is the main antigen trigger of the hyperacute rejection in humans after xenotransplantation (26). All mammals except humans, apes, and Old World monkeys, express  $\alpha$ Gal epitopes on their cells and tissues (27). The enzyme responsible for the synthesis of the  $\alpha$ Gal epitope is  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ GT). In the Golgi apparatus,  $\alpha$ GT adds a terminal galactose onto glycoproteins and glycolipids in a specific  $\alpha$ 1-3 glycosidic linkage (28). This enzyme is not transcribed in humans due to a mutation in the upstream regulatory sequence of the



$\alpha$ GT gene (26). In humans, the  $\alpha$ Gal moiety expressed by the normal bacterial flora found in intestines and lungs stimulate the production of anti- $\alpha$ Gal antibodies found in blood. These naturally acquired anti- $\alpha$ Gal antibodies constitute 1% of circulating antibodies in humans (29). After xenotransplantation, anti- $\alpha$ Gal antibodies bind to  $\alpha$ Gal epitopes on xenografts. This will then initiate complement lysis and activation through the classical pathway and natural killer (NK)-mediated antibody dependent cell cytotoxicity (ADCC) (26, 30). This is an acute response and leads to xenotransplant rejection and destruction within minutes to hours after transplantation.

### ***Development of The $\alpha$ Gal-expressing Cancer Vaccine***

Exploitation of this aspect of the hyperacute rejection of xenotransplants as a cancer therapy resulted from early studies conducted by Dr. Charles Link at the Stoddard Cancer Research Center (SCRI) in Des Moines, Iowa. In these studies, vector producer cells (VPCs) were injected into the peritoneal cavity of ovarian cancer patients. Following injection, the VPCs were destroyed by the peritoneal fluid. Other studies reported anti-tumor activity in the context of several human gene therapy cancer trials using murine retroviral VPCs (31). Those trials were based on the hypothesis that murine retroviral VPCs would efficiently transfer a suicide gene to the patient's solid tumor cells *in vivo*, thus, precipitating the subsequent destruction of tumor cells. Our team speculated that the anti-tumor responses observed in those trials were more dependent on an immune response to the  $\alpha$ Gal epitope expressed by the murine VPCs than on the gene transfer. This bystander effect might have made it possible for an entire tumor to be destroyed even though only a small portion of the tumor was affected by gene transfer. In the

ovarian cancer trial conducted at SCRI, four of ten patients demonstrated evidence of anti-tumor response, including one complete remission by CT scan. Patients showed tumor regression despite minimal transgene transfer suggesting that gene transfer was not likely to be responsible for the anti-tumor activity. An increase in anti- $\alpha$ Gal antibody titer was shown in the peritoneal cavity after VPC infusion without an increase in total IgG and IgM immunoglobulin levels, suggesting that the increased anti- $\alpha$ Gal antibody titer was the result of a specific immune response against  $\alpha$ Gal epitopes expressed by the murine VPCs. These findings correlated with the timing of the anti-tumor response observed. The patient who manifested the best and most durable anti-tumor response had a large increase in her serum titer of anti- $\alpha$ Gal antibodies (32-fold increase) and a rapid increase in the ability of her peritoneal fluid to kill murine VPCs as measured *ex vivo*. These data supported a correlation between anti- $\alpha$ Gal immunity and tumor response (32-37).

Data from this clinical trial inspired our team to propose a model to test the hypothesis of whether or not human cancer cells expressing the  $\alpha$ Gal epitope would induce an anti-tumor immunity and thus qualify as an anticancer vaccine candidate (38, 39). This hypothesis was tested in  $\alpha$ GT knockout (KO) mice (40) which, similar to humans, do not express  $\alpha$ Gal epitopes on their cells and thus provide an ideal model to study the *in vivo* immune response of  $\alpha$ Gal epitopes. These mice upon injection with rabbit red blood cells (RRBCs) (38, 41) or oral immunization with bacteria (42) produced high anti- $\alpha$ Gal antibody titers, similar to those observed in humans. The B16BL6 melanoma cell line is devoid of the  $\alpha$ Gal epitope and was transduced with a retroviral vector expressing the  $\alpha(1,3)$ GT and neomycin-resistance (NeoR) genes (B16 $\alpha$ Gal), or a

retroviral vector expressing the neomycin-resistance gene alone (B16NeoR). It was shown using a functional assay that the presence of anti- $\alpha$ Gal antibodies and complement is necessary to elicit cell lysis of  $\alpha$ Gal-expressing cells (39). Forty-five percent of mice with pre-existing anti- $\alpha$ Gal antibodies rejected a subcutaneous challenge with  $\alpha$ Gal-positive melanoma cells, remaining tumor-free for more than 80 days. In contrast, 90% of mice injected with  $\alpha$ Gal-negative B16 melanoma cells (B16Null) had developed melanoma. Similar results were obtained when this experiment was repeated (39). Mice surviving the lethal challenge of B16 $\alpha$ Gal were further protected from a subcutaneous re-challenge with B16Null. All control mice died from tumor progression while all mice injected with B16 $\alpha$ Gal survived the tumor and remained tumor-free for more than 70 days. This long-lasting anti-tumor immunity was induced by cytotoxic T cells specific for B16 melanoma cells (39).

Further modeling of metastatic melanoma using the  $\alpha$ GT KO murine model was performed. The  $\alpha$ GT KO mice were challenged with tumor, either subcutaneously to model solid melanomas, or intravenously to model disseminated melanomas, and then vaccinated with irradiated whole cells either expressing or not expressing the  $\alpha$ Gal epitope (43). In the pre-established subcutaneous model, mice vaccinated with B16 $\alpha$ Gal showed significant reduction in tumor size 30 days past tumor challenge compared to control mice. Similarly, in the pre-established pulmonary metastatic model, 30 days after the intravenous administration of tumor cells, metastases burden of mice vaccinated with B16 $\alpha$ Gal was significantly lower compared to mice vaccinated with B16NeoR or non-vaccinated mice. Importantly, all mice vaccinated with B16 $\alpha$ Gal remained tumor-free. Moreover, when T-cell mediated immunity (CD8<sup>+</sup> T cells) was adoptively transferred

from mice vaccinated with B16 $\alpha$ Gal to syngeneic mice bearing B16 melanoma, this led to a therapeutic response in 11 out of 12 mice, whereas mice of the control groups had significantly higher numbers of lung metastases. The B16 melanoma specificity of T cells harvested from  $\alpha$ Gal-vaccinated mice was also shown *in vitro*. Based in part on these data, several phase I/II clinical trials using human allogeneic cancer vaccines expressing  $\alpha$ Gal epitopes are currently open to treat patients with lung, prostate, pancreatic, and melanoma cancer.

### ***Combination Therapy Using Cancer Vaccines***

Despite the advances made in active immunotherapy, no therapeutic vaccine has yet been approved by the Food and Drug Administration. Current approaches in cancer therapy involve vaccination in combination with other therapies and these strategies are showing promises both in preclinical and clinical studies.

As reviewed recently by Schlom et al., there are primarily four different strategies of combination therapy using cancer vaccines under investigation in which some clinical efficacy has been shown (3). These are:

1. **Conventional combination therapy** in which vaccines are used with chemotherapeutic agents, such as 5-fluorouracil and docetaxel (44, 45).
2. **Multiple vaccine therapies** in which different types of vaccines are used together. For example, in an attempt to treat patients with prostate cancer, Kaufman is using vaccinia virus in combination with fowlpox virus (46).
3. **Phenotype alterations in tumor cells** in which therapeutics are used to alter the phenotype of cancer cells to make them more susceptible to T-

cell-mediated cytotoxicity. These treatments include radiation as well as chemotherapeutic agents (47).

4. **Vaccine in combination with agents that affect the host immune response** in which stimulants or inhibitors of immune regulatory cells or molecules are used. Stimulants include cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF) which has been used in clinical trials (48). Also, IL-15 and IL-7 have been described as useful adjuvants as they have the ability to enhance memory-T cell responses (3) (49). BCG vaccine and CpG motifs have also been used as immune stimulants in combination with vaccines (50, 51). Inhibition has been targeted against T regulatory cells (52-54), whereas other potential targets are immunosuppressive molecules, such as transforming growth factor- $\beta$  and IL-10 (3).

## ***IL-15***

Since the current trend in cancer vaccine field is to implement combinatory therapy trials, we proposed to explore if IL-15 and B16 $\alpha$ Gal vaccine combined therapy would increase the efficacy of our vaccine.

IL-15 is a 14-15kDa cytokine protein that signals through the  $\beta$  and  $\gamma$  chains of the IL-2 receptor in addition to the unique  $\alpha$  chain, IL-15R $\alpha$ . IL-15 binds to IL-15R $\alpha$  with high affinity and binds to IL-2/15R  $\beta$  and the common  $\gamma$  chain ( $\gamma_c$ ) heterodimer with an intermediate affinity (55, 56). IL-15 is synthesized by dendritic cells, monocytes, and fibroblasts (49, 57, 58), and IL-15R $\alpha$  is expressed by hematopoietic and parenchymal

cells (59). The intracellular trafficking of IL-15 and IL-15R $\alpha$  is unique among interleukins in that unlike other ligand/receptor complexes, they are recycled between the plasma membrane and endosomes for many days and are not targeted for degradation in lysosomes. This provides a reservoir of membrane-bound and biologically active IL-15 (60). Only IL-15R $\alpha$  is involved in IL-15/IL-15R $\alpha$  recycling (61). Through IL-15R $\alpha$  on dendritic cells and monocytes, IL-15 is trans-presented to NK cells and CD8<sup>+</sup> T cells. Only the expression of the IL-2/15R $\beta$  and  $\gamma$ c dimer is required on target cells for signaling (62). This trans-presentation is critical for the activation of NK cells, the differentiation of CD8<sup>+</sup> T cells to memory cells and the maintenance of CD8<sup>+</sup> memory T cells (49, 62, 63).

IL-15 has been studied extensively as a possible adjuvant for cancer vaccines and as a substitute for IL-2. IL-15 and IL-2 share many functions. They stimulate the proliferation of activated CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells, facilitate the induction of CTLs, induce the proliferation of and immunoglobulin synthesis by B cells, and stimulate the generation, proliferation, and activation of NK cells (49). However, IL-15 potentially supersedes IL-2 as an adjuvant for cancer vaccines, in that it is not involved in the elimination of self-reactive T cells. Also, the use of high doses of IL-2 has shown substantial toxicity in clinical trials (64, 65). In addition, IL-2 is required to maintain the competitive fitness of regulatory T (Treg) cells and for their retention in the peripheral T cell population, while no marked effects have been reported by IL-15 on Treg cells in murine models (49). However,  $\gamma$ c signals are required for the development of Treg cells and are able to compensate for the absence of IL-2 (66-69). Ideally, it is

important to control or reduce Treg cells because they reduce the induction of immune responses and thus impair the anti-tumor immune responses (66).

### ***IL-15 in Cancer Therapy***

The contribution of IL-15 to cancer immunosurveillance has been tested using different methods. IL-15 has been used as an exogenous protein (70-72), expressed by a vector (55, 73), and expressed by whole cells (74, 75). Also, transgenic mice constitutively expressing IL-15 (or IL-15R $\alpha$ ) or not expressing IL-15 (or IL-15R $\alpha$ ) have been produced to study the effects of IL-15 on cancer (60, 61).

In an adenocarcinoma study conducted by Meazza et al. (75), more than 50% of BALB/c mice subcutaneously injected with cells of the TS/A adenocarcinoma cell line expressing the IL-15 gene (TS/A IL-15) were tumor-free for at least 8 weeks, while 95% and 100% of mice injected with control cells developed tumor within 21 days of tumor challenge. Similarly, TS/A IL-15 cells were significantly less metastatic than control cells after 21 days of intravenous administration of the cells. In this study, *in vitro* cultures of splenocytes harvested from TS/A IL-15-vaccinated mice and stimulated with irradiated TS/A cells contained significantly higher concentrations of IFN- $\gamma$  than cultures with splenocytes of control mice. CD8<sup>+</sup> T cells were the major producers of this cytokine as indicated by a CD8<sup>+</sup> T cells depletion experiment *in vitro*. Furthermore, mitomycin or  $\gamma$ -irradiated TS/A IL-15 cells showed protection in 60% of mice when administered intraperitoneally to treat pre-established wild-type pulmonary adenocarcinoma tumor, while all control mice developed lung metastases after 21 days of metastasis implantation (75).

IL-15 transgenic mice (IL-15 Tg), constitutively overexpressing IL-15, showed significant protection from a subcutaneous challenge with B16F10 melanoma cells when compared with non-transgenic congenic mice (non-Tg) (76). More importantly, when these Tg mice were depleted of CD8<sup>+</sup> T cells using anti-CD8 monoclonal antibodies, B16F10 tumor growth progressed significantly when compared with mice receiving control IgG. Also, NK cells contributed to this protection as evidenced by *in vivo* NK cell depletion experiments. In another study, similar protection from MC38 colon carcinoma has been demonstrated in IL-15 transgenic mice, constitutively overexpressing IL-15. In this model, wild-type congenic mice died from pulmonary metastases within 6 weeks of tumor challenge, whereas transgenic mice survived the challenge with no metastases for more than 8 months (60). Thus, at least in mice, IL-15 can result in protection from tumor challenges.

### ***Effects of IL-15 on CD8<sup>+</sup> T Cells***

It is well established now that IL-15 enhances the maintenance and proliferation of memory CD8<sup>+</sup> T cells (49, 63, 77-81). In a study conducted by Oh et al. (82), female BALB/c mice were immunized with a vaccinia virus (VV) expressing the HIV-1 gp160 alone (vPE16), vPE16 and VV expressing human IL-2 (VV-IL-2), or vPE16 and VV expressing human IL-15 (VV-IL-15). Co-immunization with VV-IL-15 maintained relatively higher and steady levels of CD8<sup>+</sup> T cells *in vivo*, measured by *in vitro* tetramer binding assays, for at least 14 months when compared to the other groups. These data were consistent with the levels of antigen-specific CD8<sup>+</sup> T cell production of IFN- $\gamma$ . In an attempt to study the effects of IL-15 on CD8<sup>+</sup> T cells proliferation *in vivo*, vaccinated



mice were sacrificed 3 months after the last vaccination and CD8<sup>+</sup> T cells were adoptively transferred to naïve mice. After two months, antigen-specific CD8<sup>+</sup> T cells harvested from mice co-vaccinated with VV-IL-15 showed the greatest proliferation compared to CD8<sup>+</sup> T cells harvested from other groups of mice (82). In a recent *in vitro* study, human T cells transduced with a retroviral vector expressing IL-15 became immortalized and had phenotypically evolved into a pure CD8<sup>+</sup> T cells population (83).

Furthermore, the anti-tumor immunity of CD8<sup>+</sup> T cells was compromised in B16F10 melanoma-bearing IL-15KO mice, indicating the importance of endogenous production of IL-15 in maintaining the cytotoxic function of CD8<sup>+</sup> T cells (71). Of note, it has been reported that IL-15 KO mice show marked reduction in CD8<sup>+</sup>CD44<sup>hi</sup> memory T cells (49, 56). Interestingly, IL-15 enhances the proliferation of memory CD8<sup>+</sup> T cells regardless of antigen administration (77, 82, 84-86).

One of the mechanisms proposed for the IL-15 mediated anti-tumor response is its inhibition of IL-2-mediated activation-induced cell death which involves the elimination of self-reactive T cells (79). IL-15/IL-15R $\alpha$  binding is also critical for early activation of antigen-presenting cells (87). In addition, IL-15 plays a pivotal role in the proliferation and differentiation of NK cells, another important set of cytotoxic cells that have anti-cancer activity (88).

### ***The Anti-tumor Activity of CD8<sup>+</sup> T Cells***

The power of CD8<sup>+</sup> T cells to destroy tumors has been extensively demonstrated. Indeed many experiments have been done in which CD8<sup>+</sup> T cells were able to eradicate large masses of tumor *in vivo* in murine models (75, 76). *In vivo* depletion of CD8<sup>+</sup> T

cells using anti-mouse CD8 monoclonal antibodies resulted in recurrent adenocarcinoma in all syngeneic mice after a subcutaneous challenge with TS/A IL-15 cells. These effects were statistically significant, compared to mice not depleted of CD8<sup>+</sup> T cells (75). In another example using B16F10 tumor in IL-15 transgenic mice, subcutaneously administered tumors were significantly larger in mice depleted of CD8<sup>+</sup> T cells than in mice intraperitoneally injected with control IgG (76). Considerable attention has been given to these cells because they recognize and kill MHC class I-positive cells, a characteristic of most tumor cells (89). Indeed adoptive transfer to cancer patients of melanoma-specific CD8<sup>+</sup> T cells has been done with rare occurrences of serious toxicity (90, 91).

### ***B16 $\alpha$ Gal and B16IL-15 Combination Therapy for Melanoma***

The prolonged survival of cytotoxic memory CD8<sup>+</sup> T cells is desired to provide a durable protection in cancer patients receiving immunotherapy. Thus, we hypothesized that vaccination with IL-15-expressing vaccines will increase the efficacy of  $\alpha$ Gal-expressing cancer vaccines by inducing the maintenance and proliferation of memory cytotoxic CD8<sup>+</sup> T cells. We tested this hypothesis in the  $\alpha$ GT KO mouse model described above. The B16F0 melanoma cell line was transfected with a plasmid expressing human IL-15. Human IL-15 has previously shown activity in murine models (55, 70, 82, 92). Production of IL-15 was measured by ELISA and six clones expressing IL-15 were chosen for the purpose of this study. Those B16IL-15 clones were pooled, irradiated, tested for IL-15 production, and used to protect mice from a challenge with B16 melanoma in combination with B16 $\alpha$ Gal vaccine. Also, the combination therapy was

examined in therapeutic experiments where mice were first challenged with B16 melanoma tumor cells and then vaccinated. Tumor was implanted either subcutaneously or to detect any effects of the combination therapy on tumor metastases, mice had also received intravenous doses of tumor cells. The effects of vaccination in the presence of IL-15 on the cytotoxic activity of CD8<sup>+</sup> T cells was studied *in vivo* by adoptive transfer experiments to mice bearing B16 melanoma. During this study, some techniques needed to be optimized while other techniques needed to be initiated. The CFSE labeling of CD8<sup>+</sup> T cells was one of the techniques accomplished in this study and was used to detect any effects of the *in vivo* and *in vitro* administration of IL-15 on the proliferative potential of CD8<sup>+</sup> T cells.

Results in this study illustrated a trend indicating that administration of the combination therapy consisting of B16 $\alpha$ Gal plus B16IL-15 vaccination might have the potential of providing a better therapeutic approach than administering the B16 $\alpha$ Gal vaccine alone.

## MATERIALS AND METHODS

### ***Plasmid***

The plasmid hpIL-15/pEF-Neo was generously provided by Yutaka Tagaya (93). It is a 5400-bp plasmid and expresses human IL-15 under the control of the elongation factor 1  $\alpha$  (EF1 $\alpha$ ) promoter.

Propagation of the plasmid was done using heat shock transformation of chemically competent *E.coli* DH5 $\alpha$  bacteria and subsequent culture, all following standard protocols. QIAGEN Plasmid Maxi Kit was used for plasmid DNA purification, and plasmid identity was confirmed by restriction digestion analysis.

### ***Production of B16IL-15 Cell Line***

Unless otherwise stated, all culture conditions involved incubation of cells at 37°C, in 5% CO<sub>2</sub>, and in complete medium consisting of Dulbecco's Modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), L-glutamine, glucose, and sodium pyruvate. The B16F0 cell line is a spontaneous melanoma cell line derived from C57BL/6J mice, with a haplotype of H-2<sup>b/b</sup> (94). Cells of B16F0 cell line do not express the  $\alpha$ Gal epitope. Cultures of the B16F0 melanoma cell line were transfected with hpIL-15/pEF-Neo using the Lipofectamine TM 2000 reagent (Invitrogen, Carlsbad, CA, used at a ration of 1 $\mu$ g DNA: 2.5 $\mu$ L Lipofectamine TM 2000). The B16F0 cells were seeded into Petri dishes, at a density of 90% confluence in a volume of 5ml media without antibiotics. Twenty four hours post plating cells were transfected with 24 $\mu$ g DNA per plate. Following lipofection, stable transfectants were selected for by culturing

cells in a medium supplemented with Genetecin at a final concentration of 1mg/mL. Selection continued for 1 month, and medium was changed every 5 days. Clonal selection was conducted by limiting dilution and resultant clones were tested for IL-15 production by ELISA using a Human IL-15 kit (Human IL-15 Set; BD OptEATM, San Diego, CA). Concurrently cell proliferation was measured using the WST-1 reagent (Roche, Basel, Switzerland). Briefly 10 $\mu$ L of WST-1 was added to 100 $\mu$ L fresh medium, which was applied to cells after media had been collected to measure IL-15. The absorbance of the WST-1 medium was read at 440nm every 30 mins during incubation.

### ***B16 Melanoma Cell Vaccines***

Cultures of the B16F0 melanoma cell line were transduced with pHSPA, an HIV–based vector encoding the  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ GT) gene under the control of the PGK promoter (LTR-PGK- $\alpha$ GT-sinLTR). The expression, or lack thereof, of  $\alpha$ Gal epitopes on  $\alpha$ Gal-expressing melanoma cells (hereafter B16 $\alpha$ Gal) and non-transduced B16F0 control cells (hereafter B16N/V) was tested using an  $\alpha$ Gal binding lectin (FITC-labeled Griffonia simplicifolia isolectin B4 (IB4)) and detected by florescent-activated cell sorting, FACS. The B16F0, B16 $\alpha$ Gal, and B16IL-15 cells were  $\gamma$ -irradiated (200 Gy) and all vaccine lots produced were tested for Mycoplasma (MycoSensorTM PCR assay, Stratagene, La Jolla, CA) and viability using Propidium Iodide (PI) staining. All B16N/V and B16 $\alpha$ Gal cell vaccine lots were tested for  $\alpha$ Gal expression and B16IL-15 cell vaccine lots for IL-15 production by ELISA.

## ***The $\alpha$ GT KO Mouse Model***

Several mice of the original colony  $\alpha$ GT KO mice were purchased from Dr. J.B. Lowe (University of Michigan) and acted as founders of the colony at New Link Genetics (40). These early generation  $\alpha$ GT KO mice expressed both H-2 b and d haplotypes, as these mice were generated from crosses involving C57BL/6, DBA/2J, and 129sv inbred mouse strains. Subsequently  $\alpha$ GT KO mouse colonies homozygous for either haplotype H-2<sup>b/b</sup> or H-2<sup>d/d</sup> were generated at the Iowa State University Laboratory Animal Resource facility by our laboratory members. Animals used in this study were  $\alpha$ GT KO mice that are homozygous for H-2<sup>b/b</sup> haplotype (Figure 5). FITC-labeled IB4 was diluted 1:10 in serum-free medium (Lonza, Allendale, NJ) and added to peripheral blood lymphocytes isolated from mice. Cells were stained for 45 mins at 4°C in the dark, washed and analyzed by FACS. For haplotype determination, peripheral blood lymphocytes were stained with the following monoclonal antibodies (BD Pharmingen, San Diego, CA): PE-labeled anti-mouse H-2D<sup>b</sup> (clone KH95), FITC-labeled anti-mouse H-2K<sup>d</sup> (clone SF1-1.1), PE-labeled anti-mouse H-2D<sup>d</sup> (clone 34-2-12), and FITC-labeled anti-mouse H-2K<sup>b</sup> (clone AF6-88.5) and analyzed by FACS.

Upon immunization with rabbit red blood cells (RRBCs),  $\alpha$ GT KO mice develop high titers of anti- $\alpha$ Gal antibodies (38). Sera of mice immunized with RRBCs were collected to detect anti- $\alpha$ Gal antibodies by ELISA. Briefly, 96-well polyvinyl chloride (PVC) ELISA plates were coated with  $\alpha$ Gal-BSA (V-Labs, Inc., Covington, LA) at a concentration of 5 $\mu$ g/mL in carbonate buffer, pH = 9.6 (Sigma, St. Louis, MO) and incubated overnight at 4°C. The next day, plates were washed with wash buffer (1 x PBS,

pH = 4.7, 0.05% Tween 20) and blocked for 2 hr with a solution of 1%BSA (Sigma) in carbonate buffer. Four-fold serial dilutions of primary sera were prepared using wash buffer, added to coated-plates and incubated for 1 hr at room temperature. Wells were washed five times and a secondary antibody (horseradish peroxidase-labeled goat anti-mouse IgG, Chemicon, Temecula, CA) diluted 1:2000 in wash buffer, was added to the wells and incubated for 30 mins at room temperature. Plates were then washed five times and substrate solution (tetramethylbenzidine and hydrogen peroxide, Pierce, Rockford, IL), was added for 15 mins. The reaction was stopped with 2N H<sub>2</sub>SO<sub>4</sub> solution and absorbance values at 450 nm were obtained for each well using a Multiskan Spectrum plate reader (Thermo Labsystems, Franklin, MA).

### ***Animal Treatments***

Female and male, 8 to 12 weeks old,  $\alpha$ GT KO mice were used in this study. All mice received 2 or 3 intraperitoneal (i.p.) immunizations of RRBCs ( $1 \times 10^8$  cells per immunization) separated by two weeks. Two efficacy studies were carried out depending on the sequence of the therapy and tumor inoculation, those were therapeutic and preventive vaccination models. In both studies two routes of tumor challenge were used, subcutaneous tumor challenge, s.c., and intravenous tumor challenge, i.v. using non-irradiated B16N/V melanoma cells. All vaccinations were subcutaneously administered two or three times.

### ***Treatment of Pre-existing Melanoma Tumors***

After RRBCs immunizations, mice received  $5 \times 10^4$  of B16N/V cells injection in the s.c. model and  $2 \times 10^5$  cells in the i.v. pulmonary model five days before vaccination. In the s.c. model, mice were vaccinated twice, one week apart. Treatment groups, the vaccination doses per mouse, and the number of animals per group are shown in Table 1. Mice were monitored for tumor growth and survival three times a week. Tumors were measured using a digital caliper and mice with necrotic tumor and/or tumor size reaching  $1000\text{mm}^3$  were humanely euthanized. In the pulmonary model, mice were vaccinated three times, one week apart. Treatment groups, the vaccination doses per mouse, and the number of animals per group are shown in Table 2. In this pre-established pulmonary model, B16IL-15 vaccine was administered the next day. Three weeks after tumor implantation mice were sacrificed and lungs and tumors harvested and measured. All mice were treated according to the Institutional Animal Care and Use Committee (IACUC)-approved protocols.

**Table 1. Treatment of pre-established subcutaneous melanoma tumors.** The table shows the different treatments administered to mice, the vaccine dose given to each mouse, and the number of animals per group in the pre-established subcutaneous experiment.

| <b>Treatment</b>            | <b>Number of mice per group</b> | <b>Vaccine Dose (cells/mouse)</b> |
|-----------------------------|---------------------------------|-----------------------------------|
| No Treatment                | 17                              | No vaccine                        |
| B16N/V Only                 | 17                              | $1 \times 10^6$                   |
| B16N/V + B16IL-15           | 14                              | $1 \times 10^6 + 1 \times 10^6$   |
| B16 $\alpha$ Gal Only       | 18                              | $1 \times 10^6$                   |
| B16 $\alpha$ Gal + B16IL-15 | 23                              | $1 \times 10^6 + 1 \times 10^6$   |



**Table 2. Treatment of pre-existing pulmonary melanoma tumors.** The table shows the different treatments administered to mice, the vaccine dose given to each mouse, and the number of animals in the pre-existing pulmonary experiment. In this experiment, B16IL-15 vaccine was administered the day after B16N/V.

| <b>Treatment</b>            | <b>Number of mice per group</b> | <b>Vaccine Dose (cells/mouse)</b> |
|-----------------------------|---------------------------------|-----------------------------------|
| No Treatment                | 15                              | No vaccine                        |
| B16N/V Only                 | 14                              | $5 \times 10^5$                   |
| B16N/V + B16IL-15           | 14                              | $5 \times 10^5 + 1 \times 10^6$   |
| B16 $\alpha$ Gal Only       | 15                              | $5 \times 10^5$                   |
| B16 $\alpha$ Gal + B16IL-15 | 14                              | $5 \times 10^5 + 1 \times 10^6$   |

### ***Preventive Therapy Experiments***

Important modifications were done in these sets of experiments. In addition to mice receiving vaccinations about five weeks prior to tumor challenge, all treatment groups were given the same total number of cells,  $1 \times 10^6$  vaccine cells. Mice receiving B16 $\alpha$ Gal vaccine cells received B16N/V vaccine cells as well. In both the preventive s.c. and pulmonary models mice were vaccinated three times one week after the third RRBCs immunization. Table 3 shows treatment groups, the vaccination dose administered per mouse, and the number of animals per group. In the s.c. model, mice were challenged with  $8 \times 10^4$  cells and in the i.v. model, mice received  $1 \times 10^5$  tumor cells. All mice were treated humanely according to the IACUC-approved protocols and were monitored in the same way described above for the therapeutic model.

**Table 3. Preventive therapy experiments.** The table shows the different treatments administered to mice, the vaccine dose given to each mouse, and the number of animals per group in both the subcutaneous and pulmonary preventive experiments.

| Treatment                   | Vaccine Dose<br>(cells/mouse)   | Number of mice/group  |                    |
|-----------------------------|---------------------------------|-----------------------|--------------------|
|                             |                                 | Subcutaneous<br>model | Pulmonary<br>model |
| No Treatment                | No vaccine                      | 18                    | 6                  |
| B16N/V Only                 | $1 \times 10^6$                 | 17                    | 4                  |
| B16N/V + B16IL-15           | $5 \times 10^5 + 5 \times 10^5$ | 18                    | 8                  |
| B16 $\alpha$ Gal + B16N/V   | $5 \times 10^5 + 5 \times 10^5$ | 19                    | 8                  |
| B16 $\alpha$ Gal + B16IL-15 | $5 \times 10^5 + 5 \times 10^5$ | 18                    | 8                  |

### ***CD8<sup>+</sup> T Cell Purification***

CD8<sup>+</sup> T cell purification method was optimized using varying media combinations for splenic processing and CD8<sup>+</sup> T cell purification. These treatments were as follows: in Treatment 1, spleens were harvested and processed using Roswell Park Memorial Institute (RPMI) medium with 10% FBS (10%RPMI), and 0.1% Tyrode's Salt Solution (TS) (St. Louis, MO) was used for CD8<sup>+</sup> T cell purification. In Treatment 2, spleens were harvested and processed using 10%RPMI, and 0.1% RPMI was used for CD8<sup>+</sup> T cell purification. In Treatment 3, spleens were harvested and processed using 10%TS, and 0.1%TS was used for CD8<sup>+</sup> T cell purification. In Treatment 4, TS was used for both harvesting and processing spleens and CD8<sup>+</sup> T cell purification. CD8<sup>+</sup> T cell purification was done using the Dynal® Mouse CD8 Negative Isolation kit (Invitrogen). Briefly, spleens were harvested using cold medium and three spleens at a time were cut

and mashed using a sterile plunger and sieve. Cell suspension (25mL) was passed through a 40 $\mu$ M strainer into 50-mL conical tube. NycoPrep (Greiner Bio-One, Oslo, Norway) (12.5mL) at room temperature was slowly added to the bottom of the tube and centrifuged at 600g for 20 mins at room temperature with no brakes. Cells were aspirated from the middle layer and washed twice with medium, using 1500rpm for 5 mins at 4°C for centrifugation. Splenocyte concentrations were adjusted to  $1 \times 10^8$  cells/mL and CD8<sup>+</sup> T cells were isolated following the manufacturer's instructions. Purified cells were stained for CD3, CD4, CD8, and CD19 markers using phycoerythrin (PE)-labeled monoclonal antibodies (BD Pharmingen) and viability was tested using PI staining. Data were analyzed by FACS.

### ***Adoptive Transfer Experiments***

Two sets of CD8<sup>+</sup> T cell adoptive transfer experiments were carried out. In the first set, CD8<sup>+</sup> T cells donor mice were immunized three times with RRBCs two weeks apart. Ten days after the last RRBCs immunization mice were vaccinated three times, one week apart. See Table 4 for treatment groups, vaccination doses received by mice, and the number of animals per group. In these experiments, B16IL-15 vaccine was given the next day. CD8<sup>+</sup> T cells were purified using Dynal Mouse CD8 Cell Negative Isolation kit, (Invitrogen) after 3 and 7 months of vaccination and intravenously transferred to recipient mice. Recipient mice were intravenously challenged with  $1 \times 10^5$  B16N/V cells. At 3 months, CD8<sup>+</sup> T cells were adoptively transferred to mice bearing 8-day tumor and were sacrificed two weeks after CD8<sup>+</sup> T cells transfer. At 7 months, CD8<sup>+</sup> T cells were adoptively transferred to mice bearing 7-day tumor and were sacrificed 21 days after

CD8<sup>+</sup> T cells transfer. Lungs and tumors were harvested from recipient mice and weighed for treatment groups comparison.

In the second set of CD8<sup>+</sup> T cell adoptive transfer experiments, one week after the third RRBCs immunization, donor mice were vaccinated three times, one week apart. See Table 5 for treatment groups, vaccination doses received by mice, and the number of animals per group. In this experiment, all vaccines were given on the same day. Five months after the last vaccination, donor mice were sacrificed and CD8<sup>+</sup> T cells were purified and adoptively transferred (i.v.) to mice bearing 6-day tumor ( $1 \times 10^5$  B16N/V cells/mouse). Recipient mice were sacrificed 21 days after CD8<sup>+</sup> T cells transfer and lungs and tumors collected for measurements.

**Table 4. Adoptive transfer experiments (Set 1).** The table shows the different treatments administered to donor mice (donors of CD8<sup>+</sup> T cells), the vaccine dose given to each mouse, and the number of animals per group in the first set of adoptive transfer experiments where CD8<sup>+</sup> T cells were transferred to syngeneic mice bearing tumor 3 and 7 months after vaccination. B16IL-15 vaccine was administered the day after B16N/V and B16 $\alpha$ Gal vaccination.

| Treatment                   | Vaccine Dose<br>(cells/mouse)   | Number of mice/ recipient group |                                |
|-----------------------------|---------------------------------|---------------------------------|--------------------------------|
|                             |                                 | Purification<br>after 3 months  | Purification<br>after 7 months |
| No Treatment                | No vaccine                      | 11                              | 13                             |
| B16N/V + B16IL-15           | $5 \times 10^5 + 1 \times 10^6$ | 12                              | 10                             |
| B16 $\alpha$ Gal + B16N/V   | $5 \times 10^5 + 5 \times 10^5$ | 14                              | 7                              |
| B16 $\alpha$ Gal + B16IL-15 | $5 \times 10^5 + 1 \times 10^6$ | 12                              | 13                             |

**Table 5. Adoptive transfer experiments (Set 2).** The table shows the different treatments administered to donor mice (donors of CD8<sup>+</sup> T cells), the vaccine dose given to each mouse, and the number of animals per group in the second set of adoptive transfer experiments where CD8<sup>+</sup> T cells were transferred to syngeneic mice bearing tumor 5 months after vaccination.

| Treatment                   | Number of mice per group | Vaccine Dose (cells/mouse)      |
|-----------------------------|--------------------------|---------------------------------|
| No Treatment                | 7                        | No vaccine                      |
| B16N/V + B16IL-15           | 7                        | $1 \times 10^6 + 1 \times 10^6$ |
| B16 $\alpha$ Gal + B16N/V   | 7                        | $1 \times 10^6 + 1 \times 10^6$ |
| B16 $\alpha$ Gal + B16IL-15 | 7                        | $1 \times 10^6 + 1 \times 10^6$ |

### ***In Vitro CFSE Labeling and Proliferation of Lymphocytes***

To measure the effects of IL-15 on CD8<sup>+</sup> T cells proliferation, *in vitro* CFSE labeling technique was set up using CellTrace™ CFSE Cell Proliferation kit (Invitrogen). Spleens were harvested and processed using TS as described above. Lymphocytes concentration was adjusted to  $5 \times 10^7$  cells/mL and  $1 \times 10^8$  cells/mL and cells were stained with CFSE at a final concentration of 5 $\mu$ M. Cells were incubated at room temperature with continuous mixing and the reaction was stopped after five mins with cold FBS. Cells were washed three times with TS and cultured using either X-Vivo 15TM (Lonza) or RPMI 1640 complete medium at two different concentrations,  $1.5 \times 10^6$  cells/mL and  $2.5 \times 10^6$  cells/mL in 24-well plates. CD3/CD28 beads (Dynabeads® Mouse CD3/CD28 T Cell Expander, Invitrogen) were used for the proliferation of mouse T cells following manufacturer's instructions. Recombinant human IL-2 (rhIL-2) was added at a concentration of 10 IU/mL (R & D Systems, Minneapolis, MN). Cell cultures

were prepared in a final volume of 2mL and cells were incubated at 37°C and 5% CO<sub>2</sub> for three and four days. The same method was followed to trace the proliferation of CD8<sup>+</sup> T cells.

Next, CD8<sup>+</sup> T cells were purified from B16αGal + B16N/V and B16αGal + B16IL-15-vaccinated mice 2 months after vaccination, labeled with CFSE at a final concentration of 5uM, and cultured at a concentration of  $1.5 \times 10^6$  cells per mL in RPMI complete medium with  $5 \times 10^4$  irradiated B16N/V, irradiated B16N/V and IL-2 (at a concentration of 10 IU/mL), or irradiated B16IL-15 cells. Cell cultures were prepared in a final volume of 2mL in 24-well plates and cells were incubated at 37°C and 5% CO<sub>2</sub> for six days.

### ***In Vivo Proliferation of Transferred CD8<sup>+</sup> T Cells***

CD8<sup>+</sup> T cells were purified from mice vaccinated with B16αGal + B16IL-15 and B16αGal + B16N/V vaccines as described above. Purified CD8<sup>+</sup> T cells' concentration was adjusted to  $5 \times 10^7$  cells/mL and labeled with CFSE at a final concentration of 5uM as described above. CFSE-labeled CD8<sup>+</sup> T cells ( $3 \times 10^6$ ) were intravenously transferred to recipient mice. The next day, Day 0, mice were intradermally vaccinated with  $1 \times 10^6$  irradiated B16N/V melanoma cells. On days 3 and 6, recipient mice were humanely sacrificed and non-draining lymph nodes, draining lymph nodes, and spleens were harvested, processed, and lymphocytes were labeled with anti-CD3, -CD4, -CD8, and -CD19 monoclonal antibodies and analyzed by FACS. This experiment was repeated with some modifications: CD8<sup>+</sup> T cells were not only purified from two groups but from non-vaccinated mice, B16N/V-, B16αGal + B16N/V-, and B16αGal + B16IL-15-vaccinated

mice, labeled with CFSE, and intravenously transferred and lymphocytes were purified and analyzed after 6 days of stimulation.

### ***Statistical Analysis***

GraphPad Prism software was used for statistical analysis. An overall F-test was performed to test the null hypothesis of no difference between the treatment groups. Log-rank test was performed to compare animal survival curves. In addition, Student t-test was used to perform the pairwise comparisons of tumor and lung weights between the groups, and Tukey's adjustment was used to correct for multiple comparisons.

## RESULTS

### ***Section 1: Description of the plasmid, cell lines, and animal model.***

This section describes the cell lines, vaccine preparations, and the animal model used in this study.

The cell line B16F0 melanoma was used in this study. These cells were transfected with a plasmid expressing human IL-15 to generate the B16IL-15 cells. Six clones of B16IL-15 were pooled, irradiated, and used for vaccination in combination with B16 melanoma cells expressing the  $\alpha$ Gal epitope (B16 $\alpha$ Gal) and naïve B16 melanoma cells that did not express the  $\alpha$ Gal epitope (B16N/V). Mice knocked out for the  $\alpha$ GT enzyme were used in this study.

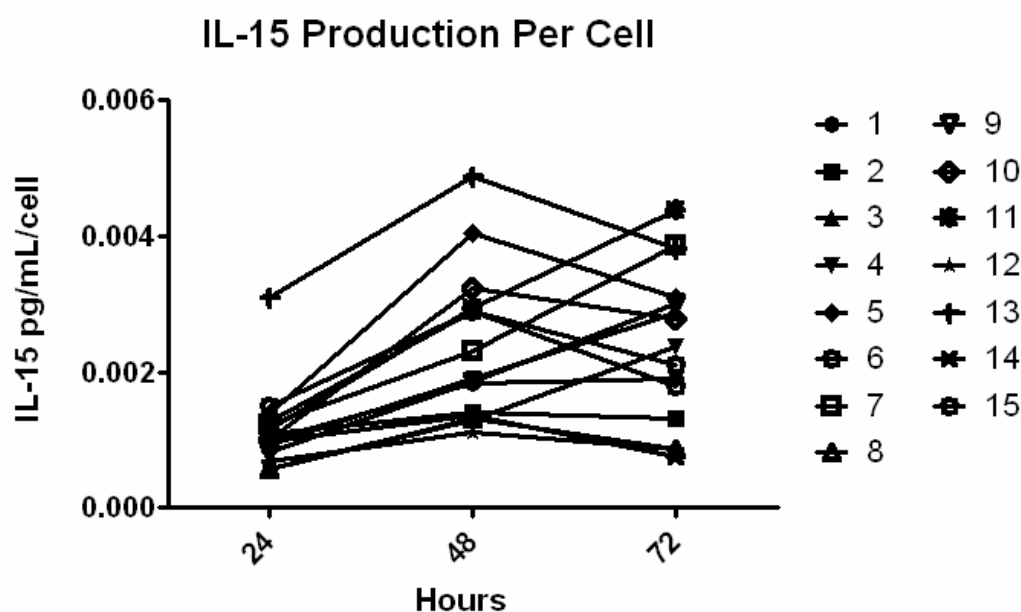
#### **Production of B16IL-15 Cell Line**

B16F0 cells were transfected with human IL-15, hereafter B16IL-15, and further screened for IL-15 production. A total of 120 clones were obtained and the production of IL-15 in the culture supernatants was tested using Human IL-15 ELISA kit (BD OptEATM,). IL-15 production of each clone was compared to IL-15 production of a mixed population of B16IL-15 cells. Clones with higher IL-15/WST-1 ratios and thus higher IL-15 production than the mixed population were chosen for further screening. WST-1 reagent is used to measure viable cells allowing the calculation of IL-15 production per cell and thus making the data of different clones comparable. From 120



clones, 15 potential clones were chosen and IL-15 production of these clones was detected after 24, 48, and 72 hr of incubation (Figure 1).

Of these potential clones, 6 clones were chosen for future experiments. Selection was based on IL-15 production rate, level, and timing. For example, Clones 7 and 9 produced IL-15 at an increasing rate compared to other clones, whereas Clone 13 was chosen because it produces IL-15 at a highest rate at all times. Clone 2 was discarded due to its low IL-15 production rate. See Figure 1. As a result, Clones 5, 7, 9, 10, 11, and 13 were chosen for further experiments.

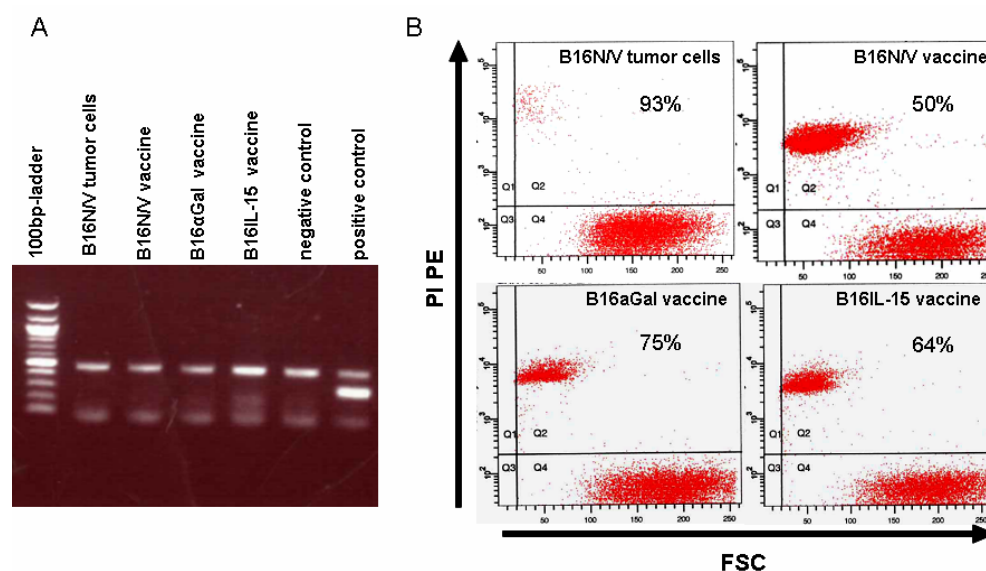


**Figure 1. IL-15 production by B16IL-15 clones.**

A total of 3000 cells were cultured in 200 $\mu$ L complete medium in 96-well plates. Each clone was represented in triplicates. Three plates were prepared for the three different incubation times: 24 hr, 48 hr, and 72 hr. Wells were checked for media every day and for ELISA 100 $\mu$ L of supernatant was used.

## B16 Melanoma Cell Vaccines

B16F0 cells (named here B16N/V), B16 $\alpha$ Gal, and B16IL-15 melanoma cells were grown in complete medium containing 1% Ciprofloxacin (1mg/mL). Five weeks after removal of antibiotic, cells received 200Gy of  $\gamma$ -irradiation and were stored in liquid nitrogen using freezing medium (90% DMSO and 10% FBS). B16IL-15 clones were grown separately and then pooled at the time of irradiation. For quality control purposes each vaccine preparation (lot) was tested for Mycoplasma using MycoSensor™ PCR assay kit from Stratagene. A representative example is shown in Figure 2A. All cells and vaccine lots used for the experiments were Mycoplasma free.

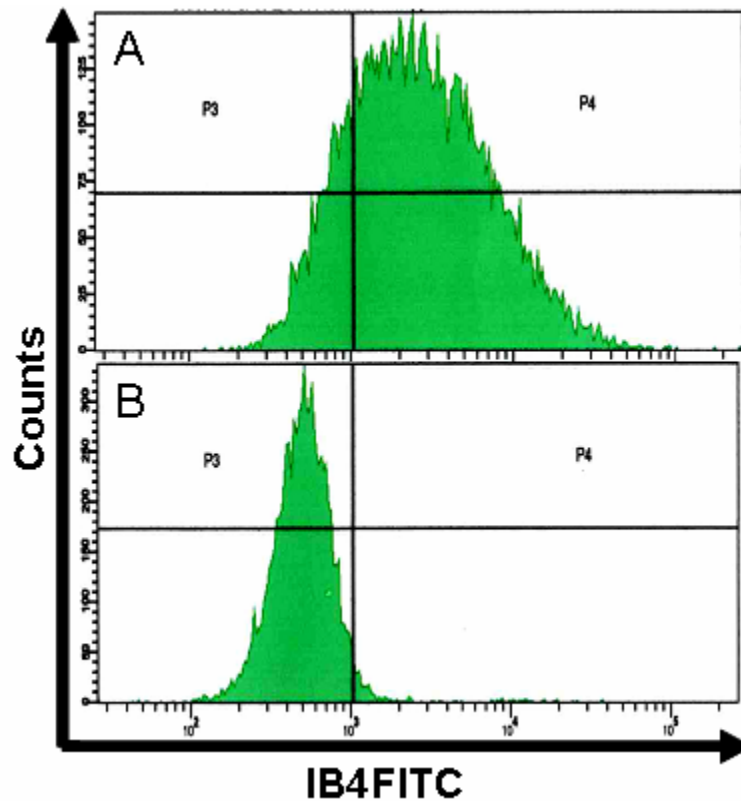


**Figure 2. B16 melanoma cell vaccines.**

A. Mycoplasma testing. MycoSensor™ PCR assay kit from Stratagene was used and manufacturer's instructions were followed. B. Viability of cells. Propidium iodide (PI) solution (5 $\mu$ L) was added to  $1 \times 10^6$  cells and data were analyzed by FACS. PI dye stains dead cells. In each panel the percentage of viable cells before vaccination is shown.

The viability of all vaccine lots and B16 cells used for tumor challenge (B16N/V tumor cells) was tested for each experiment by the incorporation of propidium iodide (PI) fluorescent dye. About 45.0% to 75.0% of vaccine cells were viable using PI staining. Figure 2B shows a representative example indicating that 93.5% of tumor cells were viable before tumor challenge, and the viability of vaccine cells was 49.6%, 74.7%, and 64.0% for B16N/V, B16 $\alpha$ Gal, and B16IL-15 vaccines, respectively.

B16N/V cells which are derived from C57BL/6 melanoma do not express  $\alpha$ Gal epitopes due to down-regulation of the  $\alpha$ GT gene (95). Nevertheless,  $\alpha$ Gal expression of transduced and non-transduced cells was tested for each vaccine lot using IB4-FITC lectin. This lectin has been shown to specifically bind to  $\alpha$ Gal epitopes (96). About 65.0% to 80.0% of B16 $\alpha$ Gal vaccine cells expressed  $\alpha$ Gal epitopes, similar to the level of  $\alpha$ Gal expression of vaccines previously used by investigators at our laboratory (39, 43) (Figure 3).

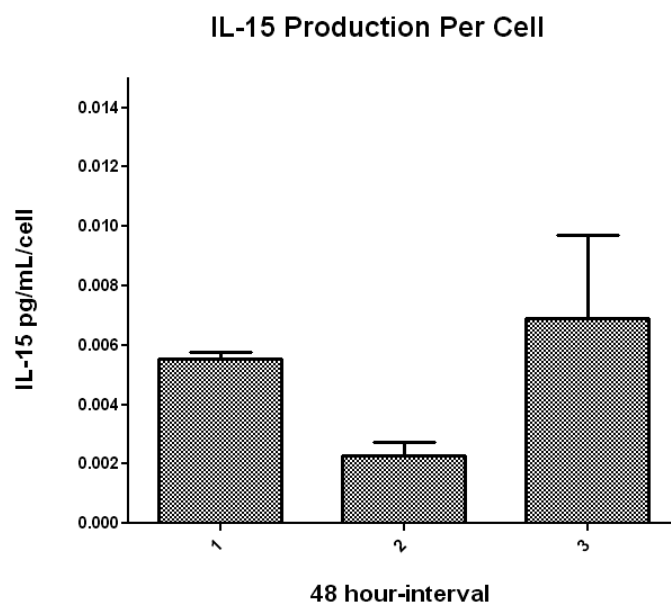


**Figure 3. Expression of  $\alpha$ Gal epitopes by vaccine cells.**

Cells ( $2 \times 10^6$ ) were stained with  $5\mu\text{L}$  of IB4-FITC lectin. After 30 mins of incubation at  $4^\circ\text{C}$ , cells were washed and analyzed by FACS. A. B16F0 melanoma cells transduced with a vector encoding  $\alpha$ GT gene (referred to in manuscript as B16 $\alpha$ Gal). B. B16F0 melanoma cells, nontransduced (referred to in manuscript as B16N/V).

B16IL-15 clones were grown separately and then pooled at the time of irradiation to prevent the outgrowth of any particular clone. After irradiation cells were tested for IL-15 production using Human IL-15 ELISA kit. We tested IL-15 production of the vaccine lots after three 48 hr-intervals. Cells were incubated in triplicates in three different 96-well plates. After 48 hr of incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , cells in the first plate were tested for IL-15 production. On that same day, the medium was changed for the second and third plates and plates were incubated for an additional 48 hr. After the second 48 hr-

incubation interval (four days of the start date of the experiment), cells of the second plate were tested for IL-15 production while the medium of the third plate was changed. The cells of the third plate were tested for IL-15 production after a third 48 hr-interval (six days after the start of the experiment). This assay was designed to test the duration of IL-15 production by B16IL-15 cell clones. On average, this is a result of triplicates of different cell concentrations, B16IL-15 cells produced  $55.5 \times 10^{-4}$  pg/mL per cell after the first 48 hr-interval. In the second interval, the mean production of IL-15 per cell was  $22.7 \times 10^{-4}$  pg/mL. In the final 48 hr-incubation period, the cells produced a mean of  $68.8 \times 10^{-4}$  pg/mL per cell (Figure 4). B16N/V vaccine cells did not produce any detectable IL-15.

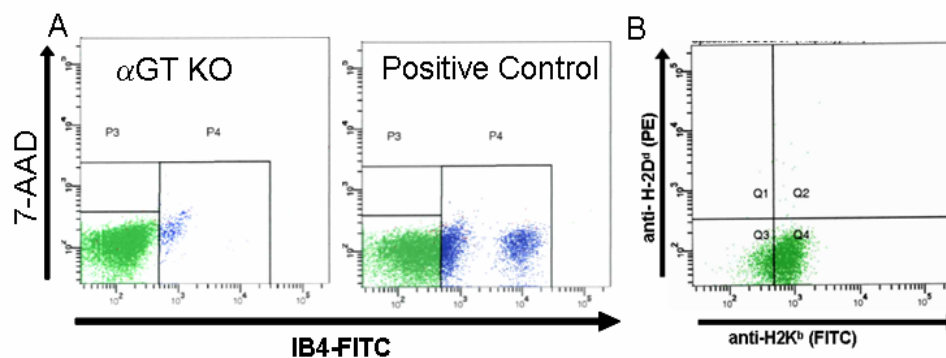


**Figure 4. IL-15 production by B16IL-15 vaccine.**

B16IL-15 vaccine cells were cultured at five different concentrations. Each concentration was evaluated in triplicates and 100µL of supernatant was harvested from each well for ELISA. Cell numbers were determined using WST-1 reagent: 100µL of fresh medium was added to the cells and then 10µL of WST-1 reagent. Cells were incubated at 37°C and 5% CO<sub>2</sub> and absorbance was measured at 440nm every 30 mins during incubation. Results shown here are calculated after three hours of incubation with WST-1 reagent.

## The $\alpha$ GT KO Mouse Model

Animals used in this study were  $\alpha$ GT KO mice originally purchased from Dr. J.B. Lowe (University of Michigan) (40). The  $\alpha$ Gal expression in these mice was tested by FITC-labeled IB4 staining using peripheral blood lymphocytes. Figure 5A shows that these cells are negative for  $\alpha$ Gal epitopes. Lymphocytes were also used to determine the haplotypes of these mice. Cells were double stained with anti-H-2K<sup>b</sup> FITC and anti-H-2D<sup>d</sup> PE monoclonal antibodies and anti-H-2K<sup>d</sup> FITC and anti-H-2D<sup>b</sup> PE monoclonal antibodies (data not shown). Cells expressed both H-2K<sup>b</sup> (Figure 5B) and H-2D<sup>b</sup> molecules and did not express H-2K<sup>d</sup> or H-2D<sup>d</sup> molecules (Figure 5B).

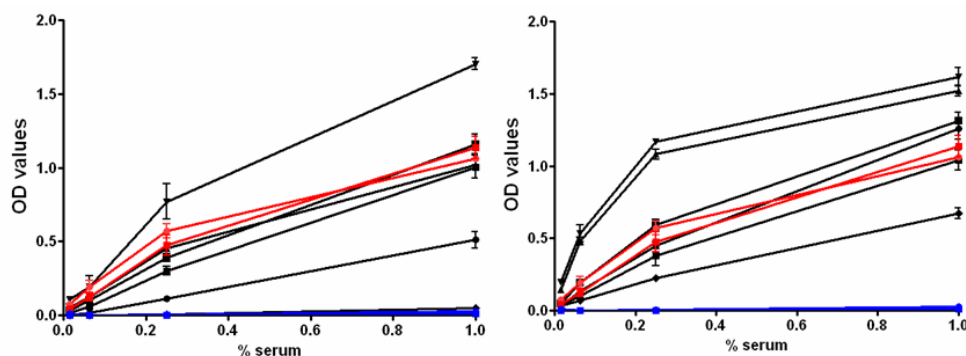


**Figure 5. Phenotype of the  $\alpha$ 1,3-galactosyltransferase knock out ( $\alpha$ GT KO) mice.**

A. Expression of  $\alpha$ Gal epitopes in  $\alpha$ GT KO mice. Peripheral blood lymphocytes isolated from  $\alpha$ GT KO mice or wild type mice (positive control) were stained with lectin and 7-AAD and analyzed by FACS. B. Mice used in this study were homozygous for H-2<sup>b/b</sup> haplotype. Peripheral blood lymphocytes were double stained with anti-H-2K<sup>b</sup> FITC + anti-H-2D<sup>d</sup> PE monoclonal antibodies and anti-H-2K<sup>d</sup> FITC + anti-H-2D<sup>b</sup> PE monoclonal antibodies (data not shown).

Humans produce natural antibodies that recognize the  $\alpha$ Gal epitope. These antibodies are often produced only in low titers in the  $\alpha$ GT KO mouse (97, 98). In order to increase the anti- $\alpha$ Gal Abs to similar levels found in humans, animals were immunized

with RRBCs. These cells express large quantities of  $\alpha$ Gal epitopes and immunization with RRBCs induces anti- $\alpha$ Gal Abs in most animals. Figure 6 shows a representative example of 12 animals used in this study immunized with RRBCs. Eleven out of 12 animals produced high titers of anti- $\alpha$ Gal Abs.



**Figure 6. Anti- $\alpha$ Gal Ab production in the  $\alpha$ GT KO mice after RRBCs immunizations.**

Mice received three RRBCs immunizations two weeks apart. Blood was collected from each mouse two weeks after the last RRBCs immunization. Four serum dilutions were prepared for each mouse sample and tested in triplicates for anti- $\alpha$ Gal IgG antibodies by ELISA. Red lines represent positive control sera and blue lines represent negative control sera.

## ***Section 2: Treatment of B16 melanoma by the combination vaccine approach in $\alpha$ GT KO mice***

This section describes the murine melanoma models utilized to test if the combination therapy consisting of B16 $\alpha$ Gal plus B16IL-15 vaccines would improve the treatment of melanoma compared to the conventional vaccine consisting of B16 $\alpha$ Gal only.

The combination therapy was tested in two melanoma models: the therapeutic model where mice were first challenged with tumor subcutaneously or intravenously and

then vaccinated, and the preventive model where mice were first vaccinated and then challenged with tumor cells either subcutaneously or intravenously.

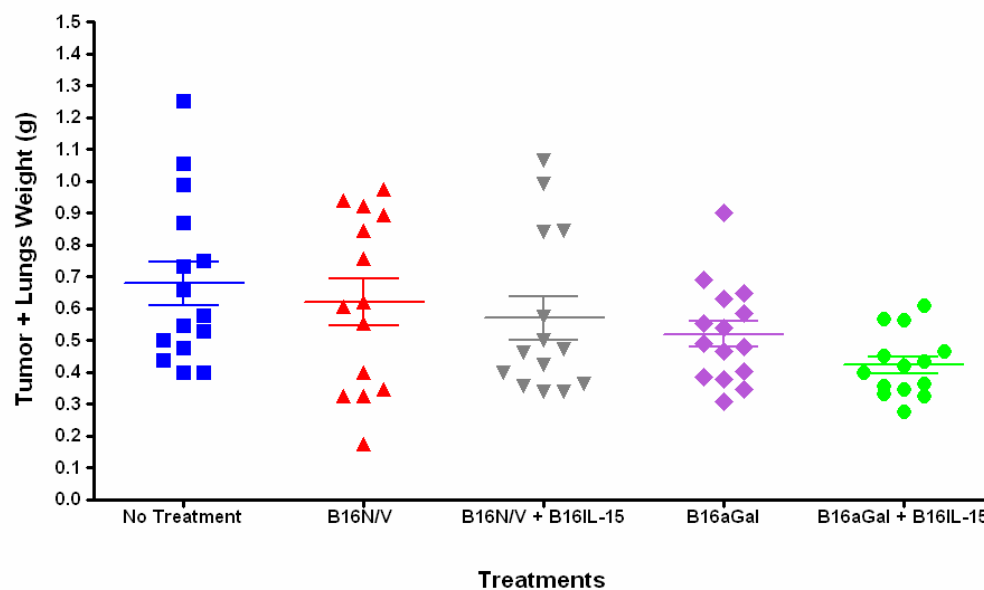
### **Treatment of Pre-existing Melanoma Tumors**

Vaccination with B16BL6 melanoma cells expressing the  $\alpha$ Gal epitope had previously shown significant reduction in the pulmonary tumors burden as well as significant enhancement of animal survival when compared to mice receiving B16BL6 vaccination only and no vaccination (43). In order to test if the addition of IL-15 to the vaccination regimen would increase the efficacy of the B16 $\alpha$ Gal vaccine, the stringency of the model was increased by reducing the therapeutic vaccine dose and by increasing the tumor implantation dose. We administered half of the B16 $\alpha$ Gal dose previously shown to be therapeutic ( $5 \times 10^5$  cells/mouse) and increased the tumor dose by two folds ( $2 \times 10^5$  cells per mouse). Under these conditions the efficacy of B16 $\alpha$ Gal vaccine was compromised in order to determine if the addition of B16IL-15 would increase vaccine efficacy.

As shown in Figure 7, treatment with B16 $\alpha$ Gal in combination with B16IL-15 significantly reduced the pulmonary melanoma metastasis burden compared to animals receiving no treatment (ANOVA  $p = 0.029$ , columns comparison by t test  $p = 0.002$ ). The addition of B16IL-15 to the B16 $\alpha$ Gal vaccine trended towards increasing the efficacy against pre-existing tumors, although this difference was not statistically significant (columns comparison by t test  $p = 0.057$ ). We compared all treatment groups using Tukey's and t tests. Results of the statistical analysis are found in Table 6.



The data suggest that the addition of B16IL-15 seems to provide a slight therapeutic benefit to the conventional B16 $\alpha$ Gal vaccine under the experimental conditions employed.



**Figure 7. Treatment of pre-existing pulmonary melanoma.**

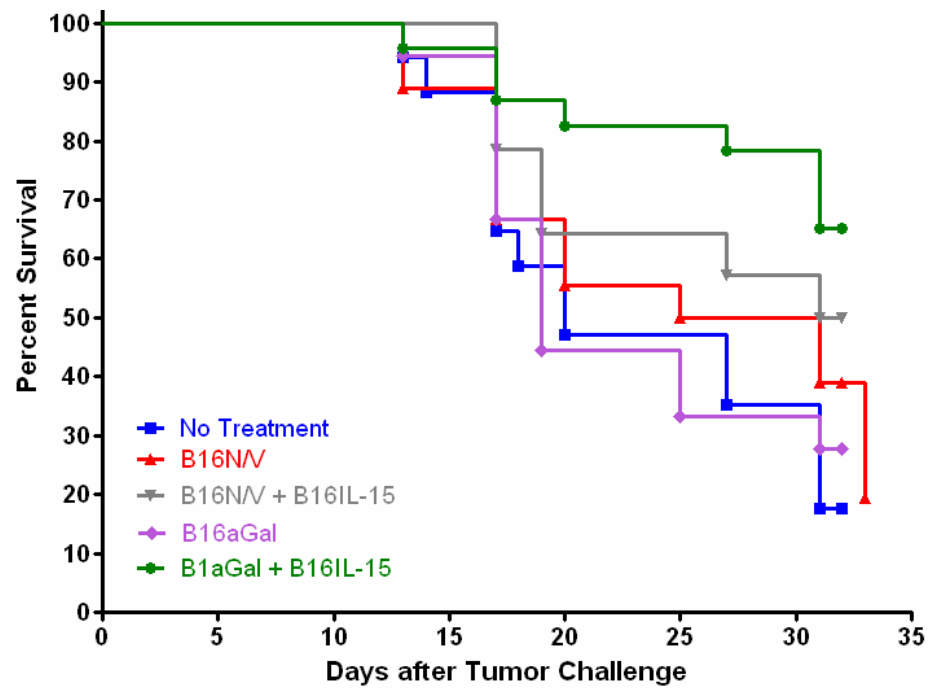
Mice received 2 intraperitoneal RRBCs immunizations ( $1 \times 10^8$  cells/mouse), subsequently they were challenged with  $2 \times 10^5$  B16N/V tumor cells intravenously. Five days later, they either received no vaccination or received 3 subcutaneous vaccinations. B16IL-15 vaccine cells were administered the day following B16N/V and B16 $\alpha$ Gal vaccinations. About two weeks after tumor challenge mice were humanely sacrificed and lungs and tumors harvested and weighed (weights are shown) for each group of mice. See Materials and Methods for the number and type of vaccine cells administered to mice in each group (Table 2).

**Table 6: Multiple columns comparison using Tukey's and t tests. Treatment of pre-existing pulmonary melanoma.** After 2 i.p. RRBCs immunizations, mice were challenged i.v. with  $2 \times 10^5$  tumor cells. Five days later they received either no vaccination or 3 s.c. vaccinations. About two weeks later, mice were sacrificed and their lungs and tumors weighed. The table shows columns comparison of the weights and highlighted are the significant p values obtained by t-test. See Materials and Methods for more details (Table 2).

| Multiple columns comparison                      | Significance by |                |
|--|-----------------|----------------|
|  | Tukey's test    | Student t test |
| No Treatment vs B16N/V                           | No              | 0.562          |
| No Treatment vs B16N/V + B16IL-15                | No              | 0.268          |
| No Treatment vs B16 $\alpha$ Gal                 | No              | 0.053          |
| No Treatment vs B16 $\alpha$ Gal + B16IL-15      | <b>Yes</b>      | <b>0.002</b>   |
| B16N/V vs B16N/V + B16IL-15                      | No              | 0.619          |
| B16N/V vs B16 $\alpha$ Gal                       | No              | 0.230          |
| B16N/V vs B16 $\alpha$ Gal + B16IL-15            | No              | <b>0.017</b>   |
| B16N/V + B16IL-15 vs B16 $\alpha$ Gal            | No              | 0.524          |
| B16N/V + B16IL-15 vs B16 $\alpha$ Gal + B16IL-15 | No              | 0.053          |
| B16 $\alpha$ Gal vs B16 $\alpha$ Gal + B16IL-15  | No              | 0.057          |

We asked the question whether this observation would be repeated in a more difficult model which is the treatment of pre-existing subcutaneous melanoma. Very few strategies thus far have demonstrated convincingly effective treatment of pre-existing subcutaneous B16 melanoma tumors (99). In this experiment, optimal vaccine doses ( $1 \times 10^6$  cells/mouse) were administered 5 days after tumor implantation. Under these conditions we demonstrated that the addition of B16IL-15 to the conventional B16 $\alpha$ Gal vaccine significantly increased the survival of vaccinated mice receiving the combination therapy (Figure 8, Log Rank test  $p = 0.008$ ). In this experiment, the B16 $\alpha$ Gal vaccine did not increase the survival of mice compared to un-treated control mice as previously shown. Currently it is unclear why this was the case. One explanation could be that the administration of vaccines was performed 5 days after tumor implantation. Previous publications using B16 cells expressing GM-CSF demonstrated that the vaccine efficacy varies tremendously depending on the timing of administration after tumor implantation. In this experiment, vaccines were administered 5 days after tumor implantation which is 2 days later than our prior work (43).

As before, multiple comparisons were made using Log Rank test (Table 7). As shown in the table the only treatment that significantly increased animal survival compared to un-treated mice was the combination therapy, B16 $\alpha$ Gal + B16IL-15 vaccine.



**Figure 8. Animal survival in the pre-established subcutaneous melanoma model.**

Mice received 3 intraperitoneal RRBCs immunizations ( $1 \times 10^8$  cells/mouse). Ten days later, they were challenged with  $5 \times 10^4$  B16N/V tumor cells subcutaneously. Five days later, mice received either no vaccination or were subcutaneously vaccinated twice, one week apart. Tumor volume was measured and mice were monitored three times a week. See Materials and Methods for the number and type of vaccine cells administered to mice and the number of animals per group (Table 1).  $P = 0.744$  for No Treatment vs B16αGal;  $p = 0.493$  for B16αGal vs B16N/V;  $p = 0.008$  for B16αGal vs B16αGal + B16IL-15;  $p = 0.001$  for No Treatment vs B16αGal + B16IL-15.

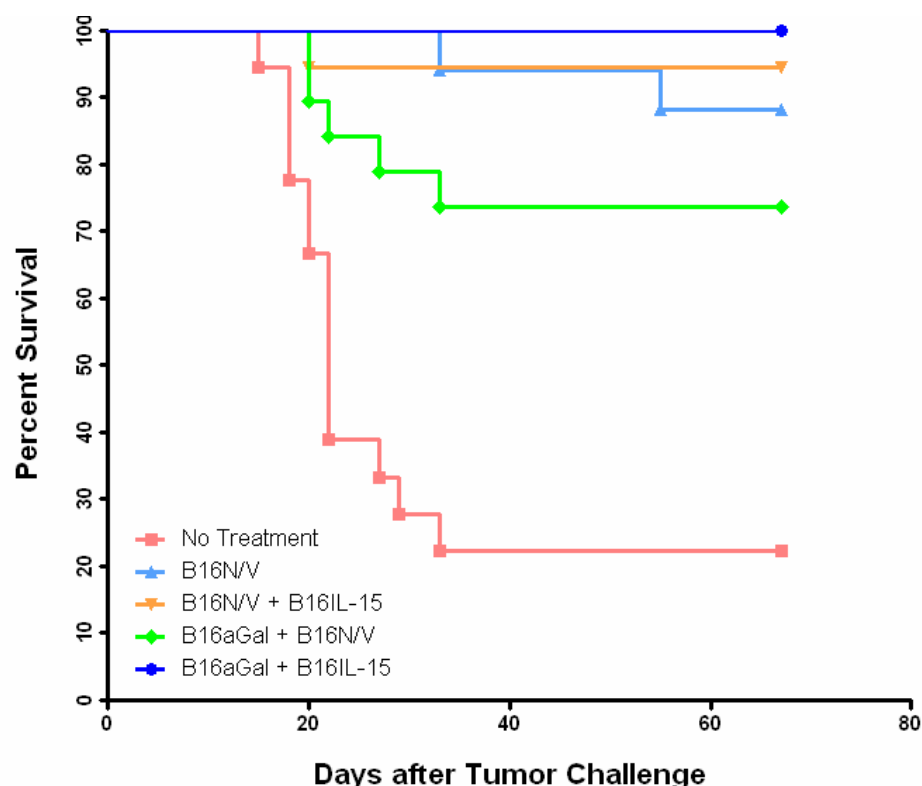
**Table 7. Multiple sets comparison using Log-Rank test. Pre-established subcutaneous melanoma model.** Ten days after the third RRBC immunization, mice were challenged s.c. with  $5 \times 10^4$  tumor cells. Five days later they received either no vaccination or 2 s.c. vaccinations. Tumor volume was measured and mice were monitored three times a week. The table shows columns comparison of animal survival 30 days after tumor challenge and highlighted are the significant p values. See Materials and Methods for more details (Table 1).

| Multiple columns comparison                      | Log Rank test |
|--|---------------|
| No Treatment vs B16N/V                           | 0.273         |
| No Treatment vs B16N/V + B16IL-15                | 0.083         |
| No Treatment vs B16 $\alpha$ Gal                 | 0.744         |
| No Treatment vs B16 $\alpha$ Gal + B16IL-15      | <b>0.001</b>  |
| B16N/V vs B16N/V + B16IL-15                      | 0.500         |
| B16N/V vs B16 $\alpha$ Gal                       | 0.493         |
| B16N/V vs B16 $\alpha$ Gal + B16IL-15            | 0.065         |
| B16N/V + B16IL-15 vs B16 $\alpha$ Gal            | 0.178         |
| B16N/V + B16IL-15 vs B16 $\alpha$ Gal + B16IL-15 | 0.312         |
| B16 $\alpha$ Gal vs B16 $\alpha$ Gal + B16IL-15  | <b>0.008</b>  |

### Preventive Therapy Experiments

We started with the pre-established melanoma model to determine whether or not B16IL-15 addition to the established system would increase the efficacy of the B16 $\alpha$ Gal treatment. However, it was not possible to determine a convincing difference among the groups in both experiments and since B16 melanoma is such an aggressive tumor model,

we decided to test the hypothesis in a less stringent model system, which is the preventative vaccination setting. In these experiments animals were vaccinated with whole cell vaccines and subsequently challenged with tumor. In addition another modification was introduced to the vaccination modality. In the previous experiments, described above, treatment groups received different cell doses of vaccines, but in the preventive experiments, mice received the same total number of vaccine cells in all treatment groups and mice receiving B16 $\alpha$ Gal vaccine received B16N/V vaccine cells as well to compensate for the B16IL-15 vaccine cells given to mice receiving B16 $\alpha$ Gal + B16IL-15 vaccine. Therefore, after eliciting anti- $\alpha$ Gal antibodies by RRBCs immunizations, mice were either subcutaneously or intravenously challenged with B16N/V tumor cells about five weeks after the third vaccination. In the subcutaneous melanoma experiment, mice were monitored for over 65 days after tumor challenge and tumor measurements were recorded three times a week. There were 5 treatment groups as mentioned in the Materials and Methods section (Table 3): non-vaccinated mice (No Treatment group), mice receiving  $1 \times 10^6$  cells of B16N/V vaccine cells alone, mice receiving  $5 \times 10^5$  cells of B16N/V and  $5 \times 10^5$  B16IL-15 vaccine cells, mice receiving  $5 \times 10^5$  B16 $\alpha$ Gal and  $5 \times 10^5$  B16N/V vaccine cells, or mice receiving  $5 \times 10^5$  B16 $\alpha$ Gal and  $5 \times 10^5$  B16IL-15 vaccine cells. All vaccinated mice showed significantly higher animal survival and reduced tumor growth when compared to non-vaccinated mice (Figure 9). In addition, there was no significant difference in animal survival or tumor growth of mice vaccinated with B16N/V alone and B16 $\alpha$ Gal + B16N/V,  $p = 0.235$ . Animal survival after 67 days of B16N/V tumor challenge was significantly higher in mice vaccinated with B16 $\alpha$ Gal + B16IL-15 than in mice vaccinated with B16 $\alpha$ Gal + B16N/V cells,  $p = 0.021$ .

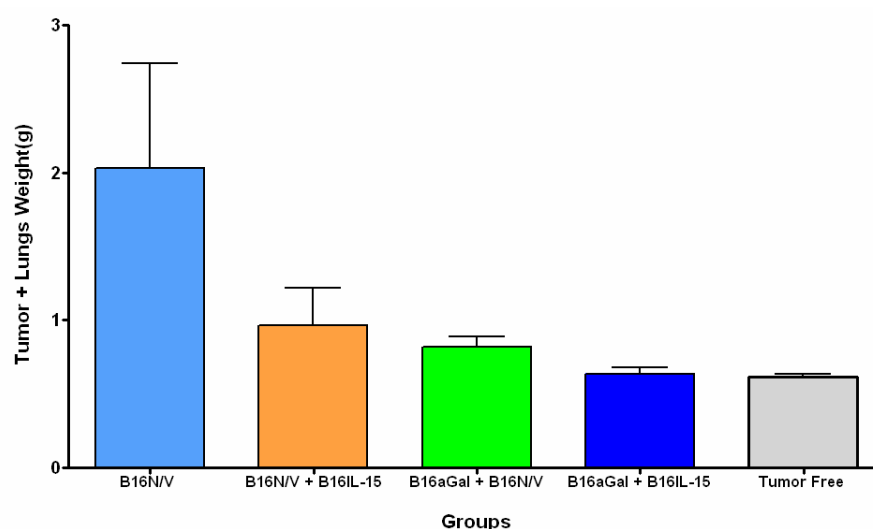


**Figure 9. Animal survival in the preventive subcutaneous melanoma model.** Mice received 3 intraperitoneal RRBCs immunizations ( $1 \times 10^8$  cells/mouse). One week later, mice received no treatment or were vaccinated three times, 2 weeks apart. Five weeks later, mice were subcutaneously challenged with  $8 \times 10^4$  B16N/V tumor cells. Tumor volume was measured and mice were monitored three times a week. See Materials and Methods for the number and type of vaccine cells administered to mice and the number of animals per group (Table 3).  $P = 0.001$  for No Treatment vs B16αGal + B16N/V;  $p = 0.235$  for B16N/V vs B16αGal + B16N/V;  $p = 0.021$  for B16αGal + B16N/V vs B16αGal + B16IL-15;  $p < 0.0001$  for No Treatment vs B16αGal + B16IL-15.

In the first preventive pulmonary experiment, male mice were sacrificed less than 4 weeks after tumor challenge as expected. Mice receiving the combination therapy, B16αGal + B16IL-15 vaccines, had significantly less tumor load than mice receiving B16αGal + B16N/V vaccines,  $p = 0.045$ . In addition, mice receiving B16αGal + B16N/V vaccine showed significantly less tumor burden than mice receiving B16N/V only,  $p =$

0.033. In this experiment, mice receiving no vaccination started dying from metastatic tumor 3 days before all mice in other groups. Thus, No Treatment group was eliminated from our data analysis. Furthermore, females of all treatment groups, except the No Treatment group, did not take the tumor as expected. They stayed alive with no signs of health deterioration due to tumor for about two months after tumor challenge. Therefore, females' data were excluded from further analysis (Figure 10). This experiment was repeated but different results were obtained. As in the preventive subcutaneous experiment, all vaccinated mice showed anti-tumor immunity against B16N/V melanoma cells but with no significant differences between any of the vaccinated groups. Tumor burden was only significantly less in vaccinated mice of each group when compared to mice of the No Treatment group,  $p < 0.05$ .





**Figure 10. Metastatic potential of B16N/V melanoma cells in the preventive pulmonary model.**

Mice received 3 intraperitoneal RRBCs immunizations ( $1 \times 10^8$  cells/mouse). One week later, mice received no treatment or were vaccinated three times, 2 weeks apart. Five weeks later, mice were intravenously challenged with  $1 \times 10^5$  B16N/V tumor cells intravenously. Three weeks after tumor challenge mice were humanely sacrificed and lungs and tumors harvested and weighed (weights are shown) for each group of mice. See Materials and Methods for the number and type of vaccine cells administered to mice and the number of animals per group (Table 3).  $P = 0.033$  for B16N/V vs B16αGal + B16N/V;  $p = 0.045$  for B16αGal + B16N/V vs B16αGal + B16IL-15;  $p = 0.016$  for B16N/V vs B16αGal + B16IL-15.

### ***Section 3: Setup experiments for CD8<sup>+</sup> T cell purification***

The purification of CD8<sup>+</sup> T cells was one of the crucial techniques used in this study. In order to recover CD8<sup>+</sup> T cells from mice with high viability and percent yield, CD8<sup>+</sup> T cell purification technique was optimized in several trials explained below.

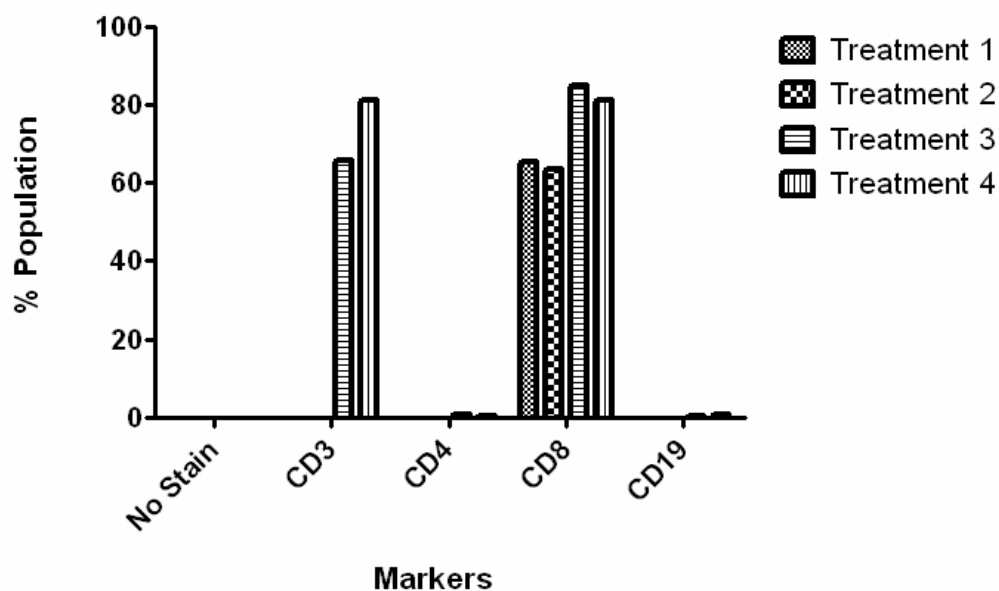
#### **CD8<sup>+</sup> T Cell Purification**

To test if IL-15 will induce the proliferation and maintenance of long term memory CD8<sup>+</sup> T cells, the technique for CD8<sup>+</sup> T cell purification needed to be perfected. These initial experiments demonstrated effective purification of CD8<sup>+</sup> T cells from non-vaccinated animals and this protocol was used in the subsequent experiments.

CD8<sup>+</sup> T cells were negatively isolated by magnetic purification from splenocytes using Dynal® Mouse CD8 Negative Isolation kit (Invitrogen) and purification efficacy was determined by FACS staining with anti-CD3, -CD4, -CD8, and -CD19 monoclonal antibodies. As described in Materials and Methods section, four treatments were tested before finalizing the best conditions for this procedure. In the first trial, Treatments 3 and 4 gave the best purification efficacy: 65.7% CD3, 84.9% CD8, and 81.2% CD3, 81.1% CD8, respectively, as well as the highest viability of cells using PI staining: 83.4% and 91.8% viable cells, respectively. Figure 11 shows all results of Trial 1. The yield of CD8<sup>+</sup> T cells using Treatments 1 and 2 was very low and there were not enough cells to stain for CD3, CD4, and CD19 markers. As a result, only Treatments 3 and 4 were chosen for further development. The purification efficacy of these two treatments did not differ significantly in any of the subsequent testing, however using only TS for harvesting and processing spleens and purifying CD8<sup>+</sup> T cells showed slightly better purification efficacy

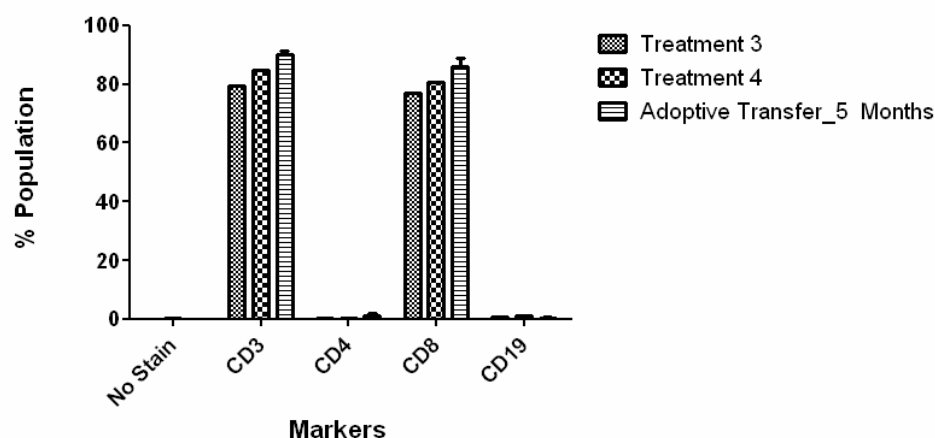
than Treatment 3 (Figure 12). Viability was also comparable for both treatments: cells treated with Treatment 3 were 94.1% viable whereas cells treated with Treatment 4 were 96.9% viable in Trial 3. Thus, TS (Treatment 4) was chosen for the second and third adoptive transfer experiments.

Figure 12 shows the results of CD8<sup>+</sup> T cell purification of the third adoptive transfer experiment, the transfer of CD8<sup>+</sup> T cells five months after vaccination (see below). On average more than 83.0% of T cells transferred to donor mice were CD3<sup>+</sup>CD8<sup>+</sup> cells and the % yield of CD8<sup>+</sup> T cells was more than 100% of expected yield.



**Figure 11. CD8<sup>+</sup> T cell purification Trial 1.**

Spleens were processed and CD8<sup>+</sup> T cells were purified from spleens using different media combinations. See Materials and Methods section for details. Cells were stained with anti-CD3, -CD4, -CD8, and -CD19 using PE-labeled monoclonal antibodies and data were analyzed by FACS.



**Figure 12. CD8<sup>+</sup> T cell purification Trial 3.**

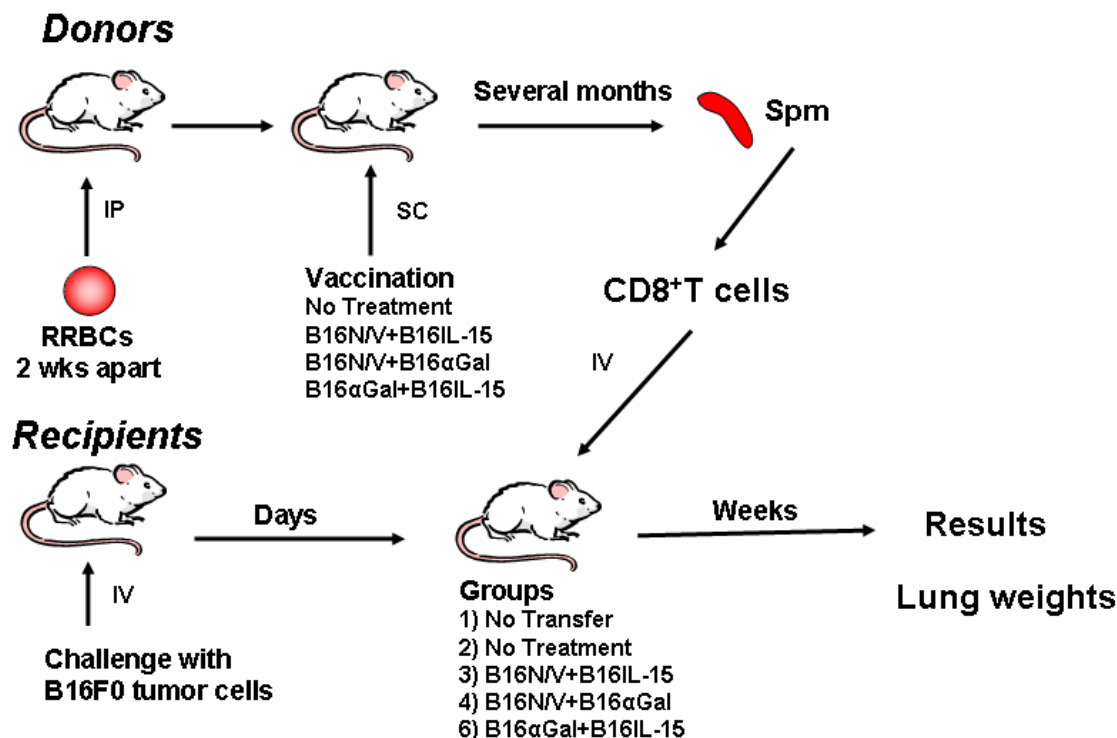
Spleens were processed and CD8<sup>+</sup> T cells were purified using Treatments 3 and 4 conditions in Trial 2 and Trial 3. See Materials and Methods for detailed description of the treatments. TS (Treatment 4) was chosen to process spleens and purify CD8<sup>+</sup> T cells for the second and third adoptive transfer experiments. The average efficacy of purifying CD8<sup>+</sup> T cells from the four groups of mice: No Treatment, B16N/V + B16IL-15, B16 $\alpha$ Gal + B16N/V, and B16 $\alpha$ Gal + B16IL-15 in the third adoptive transfer experiment is also shown. Cells were stained with anti-CD3, -CD4, -CD8, and -CD19 monoclonal antibodies and data were analyzed by FACS.

#### ***Section 4: Adoptive transfer experiments to treat pre-existing pulmonary melanoma.***

In addition to detecting any effects of combining IL-15 with B16 $\alpha$ Gal vaccine on the treatment and prevention of tumor, the effects of IL-15 on the cytotoxic activity of CD8<sup>+</sup> T cells were also tested in adoptive transfer experiments. Purified CD8<sup>+</sup> T cells were obtained from vaccinated and non-vaccinated mice and adoptively transferred to mice bearing pulmonary tumors.

##### **The Experimental Design of the Adoptive CD8<sup>+</sup> T Cell Transfer Experiments**

It was shown previously that CD8<sup>+</sup> T cells adoptively transferred from  $\alpha$ GT KO mice vaccinated with B16BL6 cells expressing the  $\alpha$ Gal epitope recognized B16BL6 tumor cells when transferred two weeks after the last vaccination to syngeneic  $\alpha$ GT KO mice bearing 5-day B16BL6 melanoma tumors (43). To test if the introduction of IL-15 to the system will enhance the maintenance of long-term memory CD8<sup>+</sup> T cells as reported in literature, CD8<sup>+</sup> T cells were adoptively transferred from non-vaccinated and vaccinated mice to recipient mice bearing tumors. Purified CD8<sup>+</sup> T cells were transferred after 3, 5, and 7 months of vaccination (Figure 13).



**Figure 13. The experimental design of adoptive transfer experiments.**

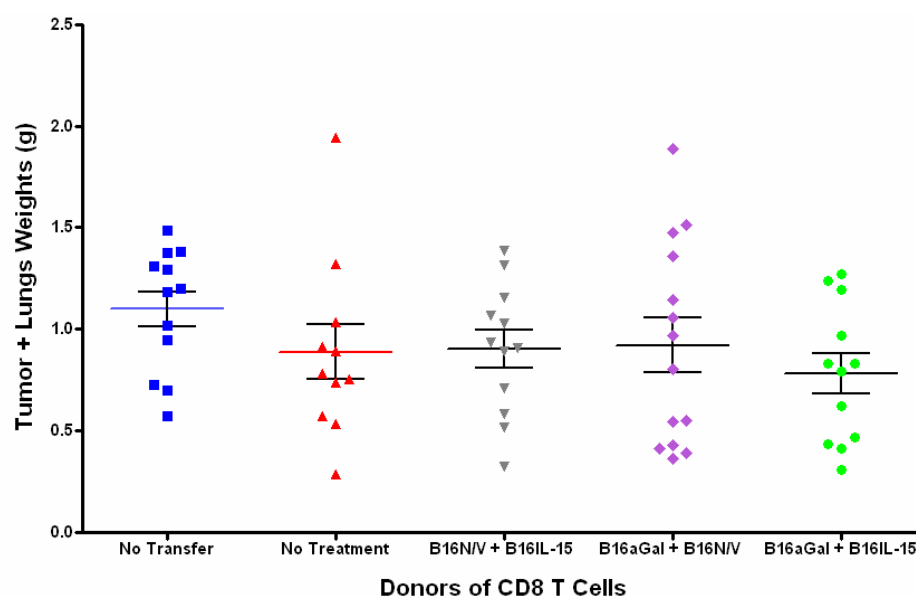
Donor mice (donors of CD8<sup>+</sup> T cells) receive the standard immunization with RRBCs, then are vaccinated or not. After resting for several months, donor mice are sacrificed and their spleens harvested. CD8<sup>+</sup> T cells are then purified from spleens and intravenously transferred to mice bearing tumor. Recipient mice are monitored and around two weeks after tumor challenge they start to show signs of discomfort due tumor burden. The experiment is then terminated and the lungs of recipient mice are harvested and weighed.

### **Transfer of CD8<sup>+</sup> T Cells from Vaccinated Mice to Recipient**

#### **Mice Bearing Pulmonary Tumors**

Mice were vaccinated as explained above and rested for several months to test for the induction of long-term memory cells after vaccination. In the first experiment, CD8<sup>+</sup> T cells were purified 3 months after vaccination and intravenously transferred to recipient mice bearing 8-day tumor. Recipient mice were humanely euthanized and lungs and

tumors harvested and weighed for groups' comparison. The only significant difference obtained was between mice that received no CD8<sup>+</sup> T cells (No Transfer group) and mice receiving CD8<sup>+</sup> T cells from donor mice vaccinated with B16 $\alpha$ Gal + B16IL-15 cells,  $p = 0.024$  (Figure 14). In this experiment and before transfer, purified CD8<sup>+</sup> T cells clumped upon resuspending with Dulbecco's Phosphate Buffered Saline 1X (DPBS) and thus the viability of the cells was compromised. Less than 50.0% of the cells were viable before transfer; this was indicated by trypan blue exclusion.



**Figure 14. CD8<sup>+</sup> T cells adoptively transferred to syngeneic mice after 3 months of vaccination.**

CD8<sup>+</sup> T cells were intravenously transferred from vaccinated and non-vaccinated mice to mice bearing 8-day tumor three months after the last vaccination. Fourteen days after CD8<sup>+</sup> T cell transfer, lungs and metastatic tumors were collected and weighed for comparison. See Materials and Methods for detailed description of the treatment groups and number of vaccine cells administered (Table 4). The only significance obtained was when comparing B16 $\alpha$ Gal + B16IL-15 with No Transfer group,  $p = 0.024$ .

In conclusion, the results obtained were less than optimal mostly due to the poor viability of transferred cells. In order to overcome this technical difficulty, different CD8<sup>+</sup>

T cell purification procedures were tested and the optimized procedure was utilized in the following experiments.

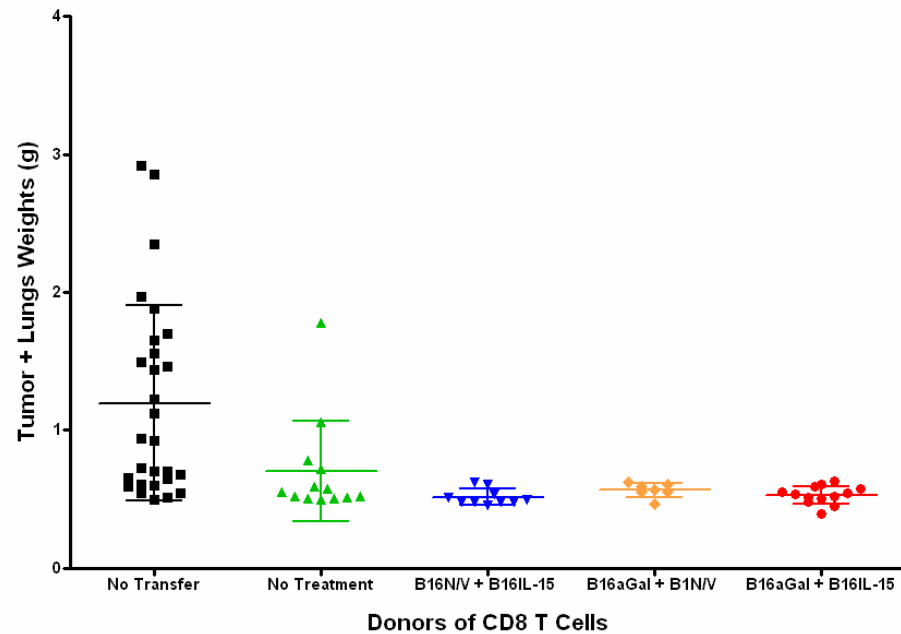
The major change in the purification procedure was the use of TS instead of PBS. Thus, for the second and third experiments, TS was used to process spleens, purify and transfer CD8<sup>+</sup> T cells (see CD8<sup>+</sup> T Cells Purification in Methods section).

Typically, pulmonary melanoma metastasis experiments are terminated when animals start to show signs of discomfort due to tumor burden. In the adoptive transfer experiment shown in Figure 15, mice receiving no CD8<sup>+</sup> T cells (No Transfer group) started to show disease symptoms, therefore the entire experiment was terminated. Unfortunately, animals receiving T cells from all other groups had very small tumors for which it was not possible to determine a statistically significant difference in the tumor burden among the groups. The only significant difference seen in this adoptive transfer experiment was between mice receiving no CD8<sup>+</sup> T cells and any other group (Figure 15). We performed a literature search to explain the reduction in melanoma tumors when CD8<sup>+</sup> T cells were transferred from non-vaccinated mice to syngeneic mice. It was demonstrated that in fact these cells induce a transient state of “immune-protection” that is subsequently lost. Only animals receiving cells from vaccinated mice showed long-term protection from melanoma (Dr Hyan Levitsky, personal communication, Cancer Vaccine Consortium Meeting 2006). Consequently, it was concluded that this T cell transfer experiment was prematurely terminated since we based our end-point on the health status (tumor burden) of animals receiving no CD8<sup>+</sup> T cells (No Transfer group).



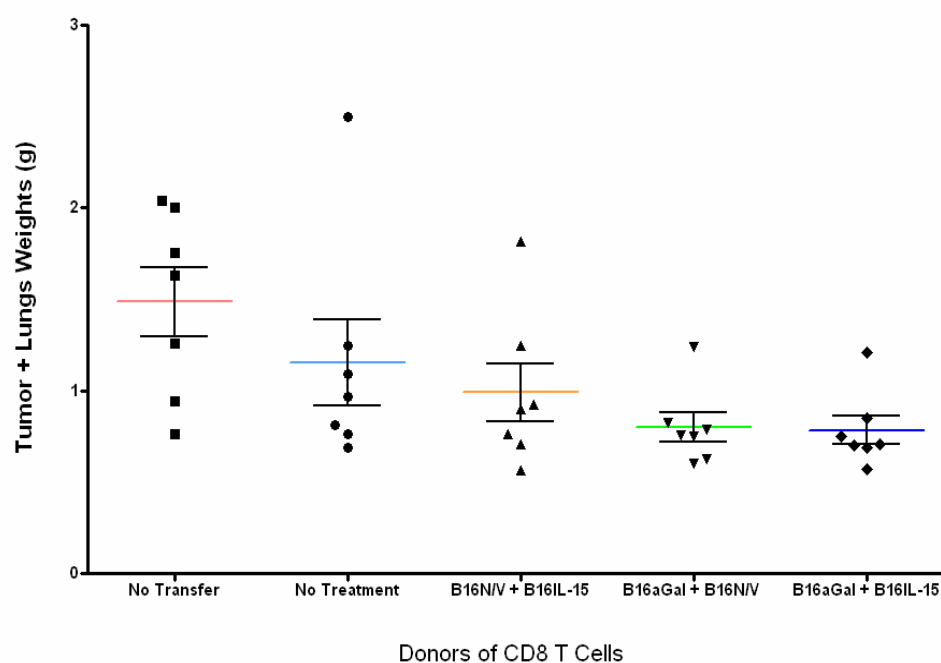
The adoptive T cell transfer experiment was repeated and based on our experience with the previous experiment it was terminated when mice receiving CD8<sup>+</sup> T cells from non-vaccinated donor mice showed signs of health deterioration due to tumor metastases. The group of animals receiving no T cell transfer was included and used as control for tumor take.

Results from this experiment showed that there was a significant reduction in tumor burden when comparing mice receiving either B16 $\alpha$ Gal + B16N/V or B16 $\alpha$ Gal + B16IL-15 with mice receiving no CD8<sup>+</sup> T cells, No Transfer group,  $p = 0.006$  and  $p = 0.005$ , respectively. There was no significant difference in tumor burden between mice receiving B16 $\alpha$ Gal + B16IL-15 and mice receiving B16 $\alpha$ Gal + B16N/V,  $p = 0.885$  or when comparing the later groups with mice receiving CD8<sup>+</sup> T cells from non-vaccinated mice. As in a previous experiment, females did not develop tumor upon challenge with B16N/V tumor cells and thus their data were excluded from the analysis. Figure 16 shows only male mice data.



**Figure 15. CD8<sup>+</sup> T cells adoptively transferred to syngeneic mice after 7 months of vaccination.**

CD8<sup>+</sup> T cells were intravenously transferred from vaccinated and non-vaccinated mice to mice bearing 7-day tumor 7 months after the last vaccination. Twenty one days after CD8<sup>+</sup> T cells transfer, lungs and metastatic tumors were collected and weighed for comparison. See Materials and Methods for detailed description of the treatment groups and number of vaccine cells administered (Table 4). There was a significant reduction in tumor burden when comparing any treatment group with the No Transfer group,  $p < 0.05$ .



**Figure 16. CD8<sup>+</sup> T cells adoptively transferred to syngeneic mice after 5 months of vaccination.**

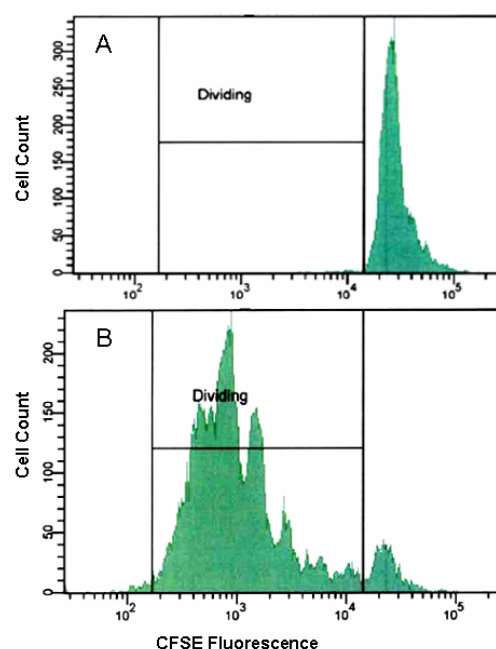
CD8<sup>+</sup> T cells were intravenously transferred from vaccinated and non-vaccinated mice to mice bearing 6-day tumor 5 months after the last vaccination. Twenty one days after CD8<sup>+</sup> T cells transfer, lungs and metastatic tumors were collected and weighed for comparison. See Materials and Methods for detailed description of the treatment groups and number of vaccine cells administered (Table 5). The only significance obtained was when comparing either B16αGal + B16N/V ( $p = 0.006$ ) or B16αGal + B16IL-15 ( $p = 0.005$ ) with the No Transfer group.

## **Section 5: Setup experiments for T cell proliferation and tracking by CFSE**

The CFSE dye was used to label T cells to monitor their proliferation *in vitro* and to locate cells *in vivo*. This technique was important to set up to detect any effects of IL-15 on the proliferation of CD8<sup>+</sup> T cells.

### ***In Vitro* CFSE Labeling and Proliferation of Lymphocytes**

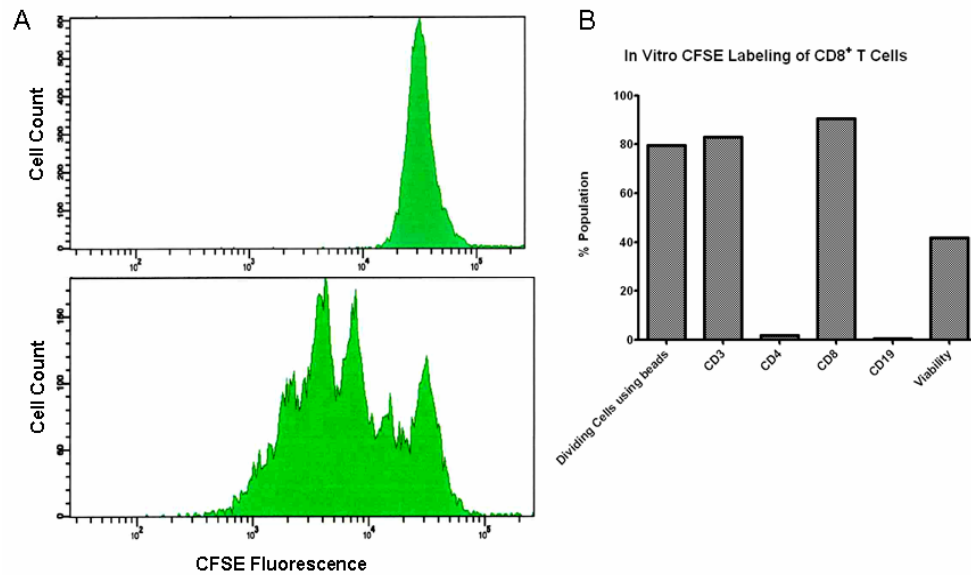
*In vitro* CFSE labeling of lymphocytes was setup in order to detect if the *in vivo* administration of vaccines expressing IL-15 would enhance the proliferative potential of CD8<sup>+</sup> T cells. Several test conditions were employed. Lymphocytes were isolated, stained with CFSE at a final concentration of 5 $\mu$ M and activated using CD3/CD28 beads. Cells were stained with CFSE at 5 x 10<sup>7</sup> cells/mL and 1 x 10<sup>8</sup> cells/mL and cultured using two different media, RPMI 1640 complete medium and X-Vivo, at two different concentrations, 1.5 x 10<sup>6</sup> cells/mL and 2.5 x 10<sup>6</sup> cells/mL in 24-well plates and in a final volume of 2mL. Cultures were incubated for 3 and 4 days at 37°C and 5% CO<sub>2</sub>. Cells under all conditions had successfully proliferated and proliferation was efficiently detected by CFSE labeling at days 3 and 4 (Figure 17). Some samples showed 93.0% proliferation and 5 to 6 cell divisions. However, the viability of cells cultured with X-Vivo was relatively low compared with cells cultured with RPMI 1640 complete medium: 52.0% and 73.0% viable cells, respectively. Thus, RPMI 1640 was chosen for further CFSE-labeling assays. The viability of cells was measured using 7-Amino-actinomycin D (7-AAD) viability staining solution from BD Pharmingen.



**Figure 17. *In vitro* CFSE labeling and proliferation of lymphocytes.**

Lymphocytes were stained with CFSE at a final concentration of  $5\mu\text{M}$  and cultured with RPMI 1640 complete medium or X-Vivo at  $1.5 \times 10^6$  cells/mL and  $2.5 \times 10^6$  cells/mL. After 3 and 4 days of culture, cells were harvested and washed once with TS and samples were analyzed by FACS. Shown here are the FACS results for cells stained with CFSE at  $5 \times 10^7$  cells/mL concentration, cultured at  $1.5 \times 10^6$  cells/mL in RPMI 1640 complete medium for four days. A. Cells cultured without CD3/CD28 activating beads. B. Cells cultured with CD3/CD28 activating beads.

The next step was to CFSE label and detect the proliferation of purified  $\text{CD8}^+$  T cells *in vitro*.  $\text{CD8}^+$  T cells were labeled with  $5\mu\text{M}$  CFSE at a concentration of  $5 \times 10^7$  cells/mL and cultured at  $1.5 \times 10^6$  cells/mL in a final volume of 2mL. After three days in culture, cells were harvested, washed once with TS, and labeled with anti-CD3, -CD4, -CD8, and -CD19 monoclonal antibodies and data were analyzed by FACS. Almost 80.0% of the cells divided of which 41.0% were viable (Figure 18).

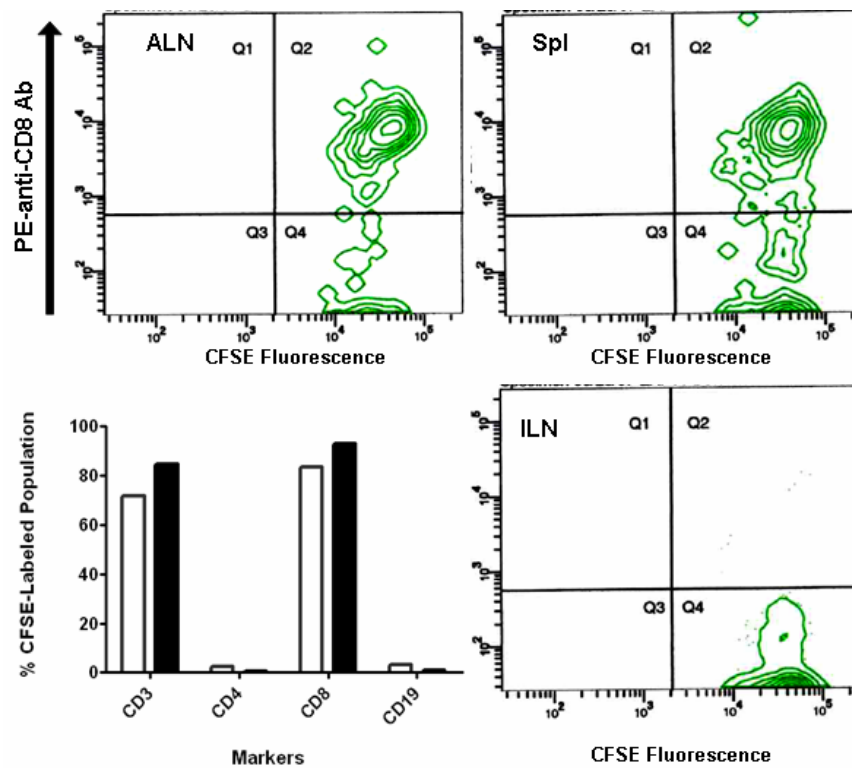


**Figure 18. *In vitro* CFSE labeling and proliferation of CD8<sup>+</sup> T cells.**

CD8<sup>+</sup> T cells were stained with 5 $\mu$ M CFSE at a concentration of  $5 \times 10^7$  cells/mL and then cultured at a concentration of  $1.5 \times 10^6$  cells/mL at 37°C and 5% CO<sub>2</sub> for 3 days. A. FACS results for CFSE labeling. B. Cells were stained for CD3, CD4, CD8, and CD19 markers and viability was tested by 7-AAD.

### Initial Experiment for the Transfer of CFSE-Labeled T Cells

The goal of this experiment was to determine whether transferred CFSE-labeled cells could be located. This experiment represents the first step towards determining if *in vivo* proliferation of transferred CD8<sup>+</sup> T cells occurs. As shown in Figure 19, CFSE-labeled CD8<sup>+</sup> T cells could be located in spleens, auxiliary lymph nodes, and inguinal lymph nodes in less than 48 hr after CFSE-labeled CD8<sup>+</sup> T cells transfer.



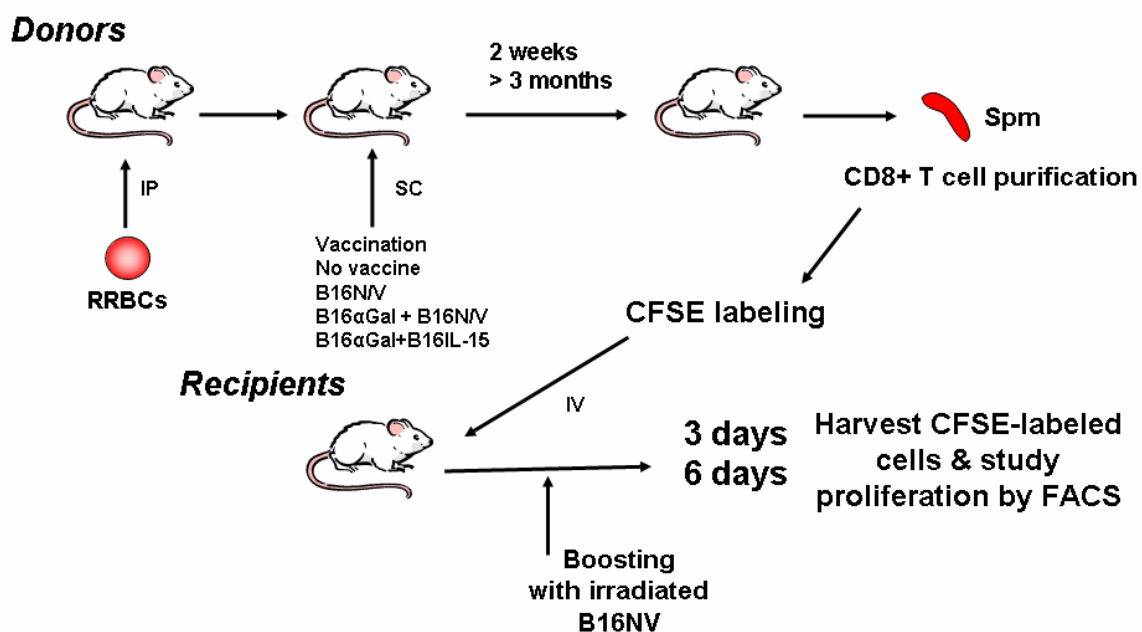
**Figure 19. Initial experiment for *in vivo* tracking.**

Shown are the results of a setup experiment to detect CFSE- labeled CD8<sup>+</sup> T cells in spleens (Spl), inguinal lymph nodes (ILN), and auxiliary lymph nodes (ALN) in less than 48 hr after their transfer. CD8<sup>+</sup> T cells were purified from mice receiving no treatments and labeled with CFSE at a final concentration of 5 $\mu$ M. CFSE-labeled CD8<sup>+</sup> T cells ( $2 \times 10^6$ ) were intravenously transferred to mice and in less than 48 hr, recipient mice were euthanized and lymphocytes purified from spleens and inguinal and auxiliary lymph nodes, and analyzed for CFSE label by FACS. ALN: Lymphocytes purified from auxiliary lymph nodes and labeled with PE-anti-CD8 monoclonal antibody; Spl: Lymphocytes purified from spleens and labeled with PE-anti-CD8 monoclonal antibody; ILN: Lymphocytes purified from inguinal lymph nodes; Lymphocytes purified from mice receiving CFSE-labeled CD8<sup>+</sup> T cells were further labeled with PE-labeled anti-CD3, -CD4, -CD8, and -CD19 monoclonal antibodies. There were not enough cells from inguinal lymph nodes to label for markers. □: Lymphocytes purified from spleens; ■: Lymphocytes purified from auxiliary lymph nodes.

## ***Section 6: Proliferation of CD8<sup>+</sup> T cells purified from vaccinated mice.***

Thus far it was demonstrated that the combinatory therapy had shown an overall benefit over the administration of B16 $\alpha$ Gal vaccine alone both in the efficacy studies and the adoptive transfer experiments. Another mode of IL-15 action that was tested in this study was the effects of IL-15 on the proliferation of CD8<sup>+</sup> T cells. The question addressed was whether or not vaccination with B16IL-15 in combination with B16 $\alpha$ Gal vaccine would improve the maintenance of memory CD8<sup>+</sup> T cells by enhancing their proliferation. Thus, CD8<sup>+</sup> T cells were isolated from vaccinated mice, labeled with CFSE, and their proliferation traced *in vitro* or *in vivo*. In the *in vivo* experiment, labeled CD8<sup>+</sup> T cells were adoptively transferred to syngeneic mice. Recipient mice were then stimulated with irradiated B16 melanoma cells (B16N/V) and sacrificed at different time points. CFSE-labeled CD8<sup>+</sup> T cells were then harvested from recipient mice and CFSE intensity was analyzed by FACS. Figure 20 describes the experimental design.



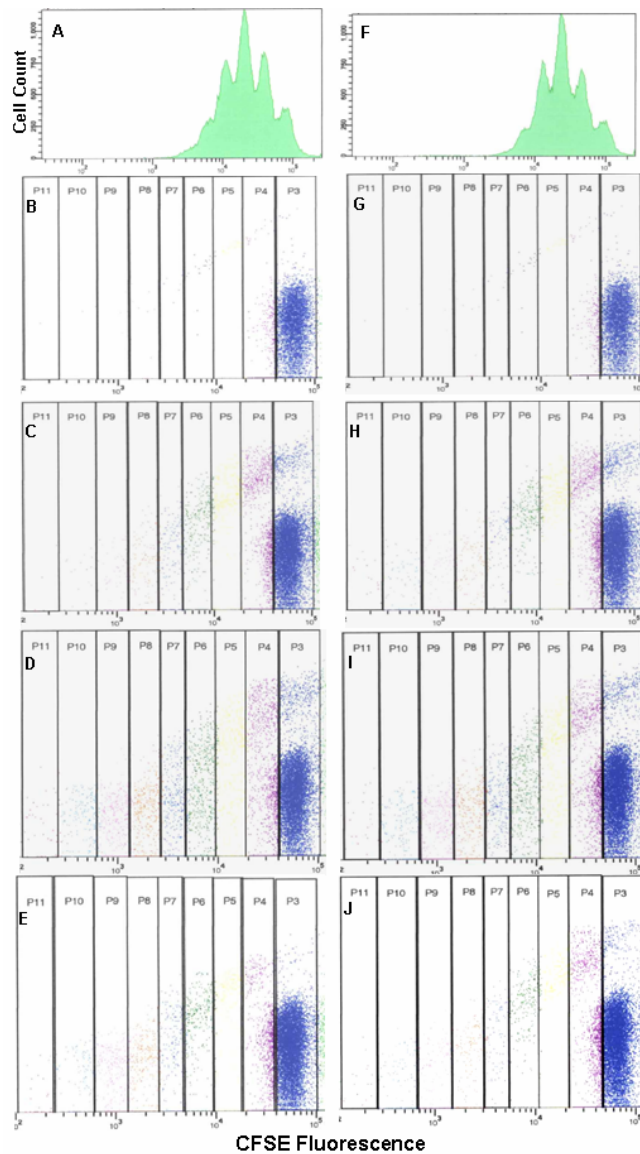


**Figure 20. The experimental design of *in vivo* proliferation of CFSE-labeled CD8<sup>+</sup> T cells.**

CD8<sup>+</sup> T cells are purified from vaccinated and non-vaccinated mice, labeled with CFSE, and adoptively transferred to syngeneic mice. The next day, recipient mice are subcutaneously vaccinated with irradiated B16N/V cells to stimulate the transferred CD8<sup>+</sup> T cells. Three and six days after stimulation mice are sacrificed and spleens are collected and processed. Lymphocytes are then analyzed by FACS to determine proliferation by CFSE intensity.

The effects of B16IL-15 vaccination on the proliferation of CD8<sup>+</sup> T cells were first tested *in vitro*. CD8<sup>+</sup> T cells were purified two months after mice were vaccinated with B16αGal + B16N/V vaccine or B16αGal + B16IL-15 vaccine. CD8<sup>+</sup> T cells were stimulated *in vitro* with irradiated B16N/V cells in the presence or absence of IL-2 or with irradiated B16IL-15 cells. All CD8<sup>+</sup> T cells showed proliferation when data were analyzed by FACS six days after stimulation (Figure 21). Under these conditions, it was not possible to detect any effects of the *in vivo* administration of IL-15 on the proliferation of CD8<sup>+</sup> T cells. In addition,

CD8<sup>+</sup> T cells were cultured with  $5 \times 10^4$  irradiated B16IL-15 cells and on average that number of cells was expected to produce 250pg/mL of IL-15 every 48 hr, nevertheless the *in vitro* presence of IL-15 did not add any effects to the proliferation of CD8<sup>+</sup> T cells compared to other stimulators (Figure 21).



**Figure 21. *In vitro* proliferation of stimulated CFSE-labeled CD8<sup>+</sup> T cells.**

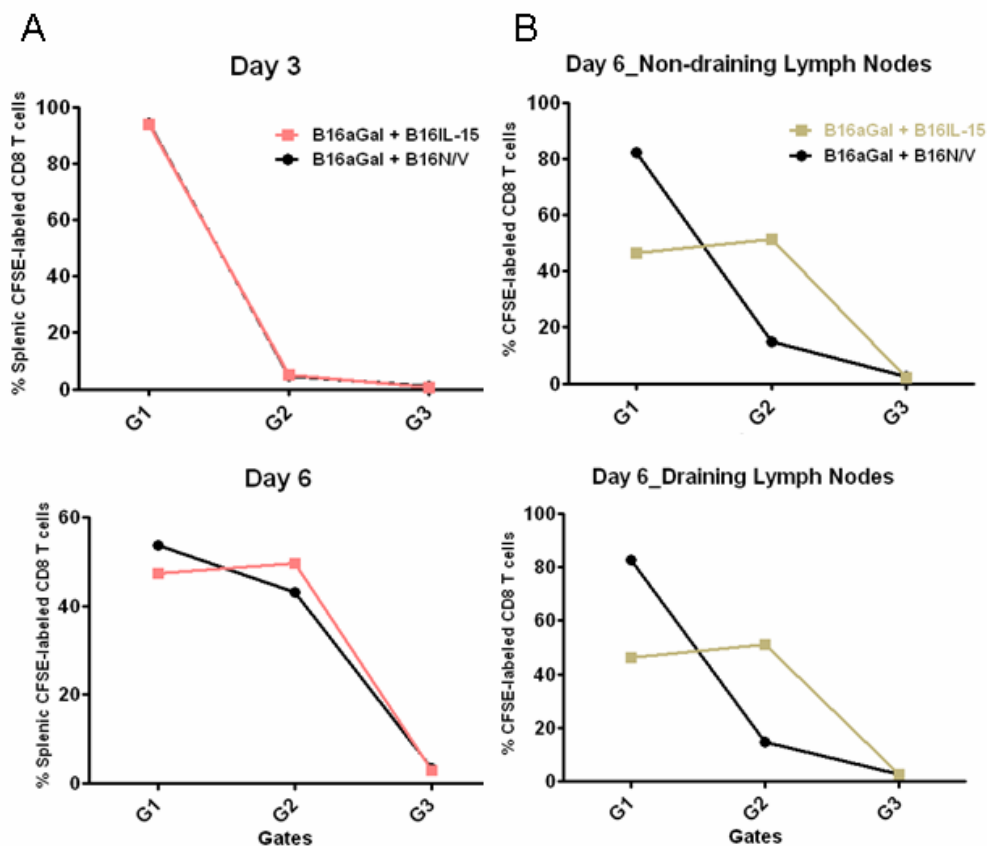
CD8<sup>+</sup> T cells were isolated from mice vaccinated with B16αGal + B16N/V (A-E) and mice vaccinated with B16αGal + B16IL-15 (F-J), labeled with CFSE and cultured at a concentration of  $1.5 \times 10^6$  cells/mL in 24-well plates in a final volume of 2mL RPMI 10. CD8<sup>+</sup> T cells were either stimulated with irradiated B16N/V cells (C + H), irradiated B16N/V cells and IL-2 (D + I), or irradiated B16IL-15 cells (E + J) for six days. A + F represent positive controls (stimulation with CD3/CD28 beads) and B + G represent negative controls (no stimulation).

### ***In Vivo* Proliferation of Transferred CD8<sup>+</sup> T Cells**

The goal of this experiment was to measure the *in vivo* proliferative potential of CD8<sup>+</sup> T cells transferred to syngeneic recipient animals. The donors of this experiment were mice that survived the subcutaneous challenge with B16N/V melanoma in the preventive experiment described above. These animals were used because they survived a lethal challenge with B16 melanoma strongly suggesting that they had developed anti-melanoma T cell reactivity.

CD8<sup>+</sup> T cells were purified from mice vaccinated with B16 $\alpha$ Gal + B16N/V and B16 $\alpha$ Gal + B16IL-15 vaccines then labeled with CFSE and intravenously transferred to recipient mice. The next day, Day 0, mice were stimulated with  $1 \times 10^6$  irradiated B16N/V cells intradermally. On Days 3 and 6, recipient mice were euthanized and lymphocytes from non-draining lymph nodes, draining lymph nodes, and spleens were purified and analyzed by FACS. Cells were gated according to their CFSE intensity; Gate (G) 1, G2, and G3 represent cells with no, one, or two cell divisions *in vivo*, respectively. There was no difference in proliferation between lymphocytes purified from non-draining and draining lymph nodes within each group (data not shown). There was no difference in the proliferation of splenic CD8<sup>+</sup> T cells purified from mice vaccinated with B16 $\alpha$ Gal + B16N/V and those purified from mice vaccinated with B16 $\alpha$ Gal + B16IL-15 on Day 3 (Figure 22A). However, on Day 6, splenic CD8<sup>+</sup> T cells purified from mice vaccinated with B16 $\alpha$ Gal + B16IL-15 showed more proliferation than those purified from mice vaccinated with B16 $\alpha$ Gal + B16N/V (Figure 22A). Also on Day 6, CD8<sup>+</sup> T cells purified from mice vaccinated with B16 $\alpha$ Gal + B16IL-15 showed more proliferation in the non-draining and draining lymph nodes of recipient mice than those purified from B16 $\alpha$ Gal +

B16N/V-vaccinated mice (22B). This result suggests that CD8<sup>+</sup> T cells induced in the presence of IL-15 demonstrated an increased proliferative potential after *in vivo* stimulation.



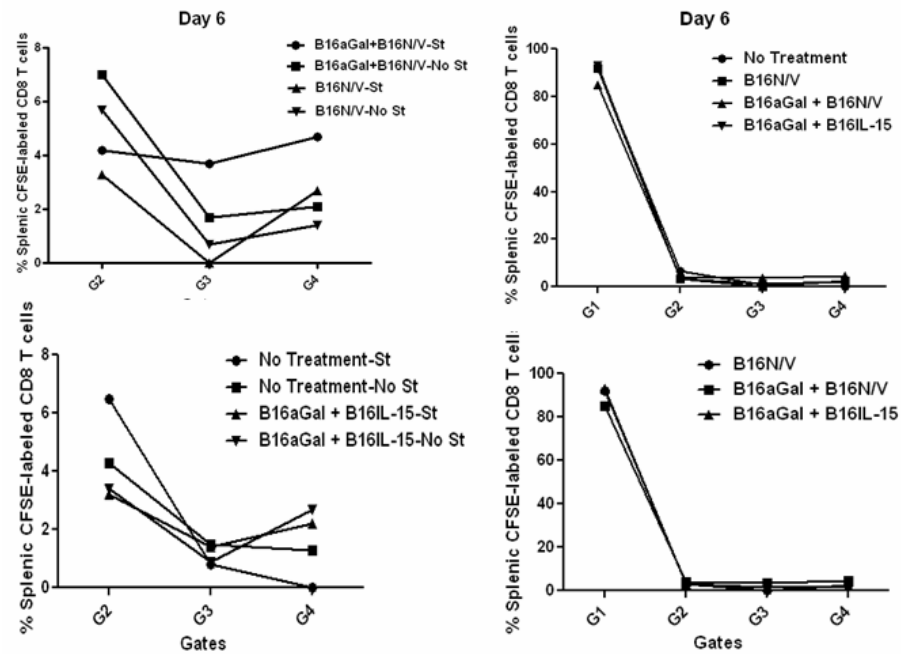
**Figure 22. *In vivo* proliferation of transferred CD8<sup>+</sup> T cells.**

CD8<sup>+</sup> T cells were purified from mice vaccinated with B16aGal + B16N/V vaccine and mice vaccinated with B16aGal + B16IL-15 vaccine, labeled with CFSE and then intravenously transferred to recipient mice. Lymphocytes were purified from recipient mice on Days 3 and 6 after stimulation with irradiated B16N/V cells. A. Purified lymphocytes from spleens were labeled with anti-CD8 monoclonal Ab. B. Transferred CD8<sup>+</sup> T cells purified from B16aGal + B16IL-15-vaccinated mice showed a higher percentage in Gate 2 than CD8<sup>+</sup> T cells purified from B16aGal + B16N/V-vaccinated mice in the non-draining and draining lymphocytes. Cells were gated according to their CFSE intensity; G1, G2, and G3 represent cells with no, one, or two cell divisions *in vivo*, respectively.

This experiment was repeated with some modifications. More groups of mice and other controls were included in this trial. Mice received three RRBCs immunizations and were then vaccinated with  $1 \times 10^6$  B16N/V cells,  $5 \times 10^5$  B16 $\alpha$ Gal +  $5 \times 10^5$  B16N/V cells or  $5 \times 10^5$  B16 $\alpha$ Gal +  $5 \times 10^5$  B16IL-15 cells and from mice that were not vaccinated. Fourteen days after the third vaccination, mice were euthanized and CD8<sup>+</sup> T cells were purified from each group, labeled with CFSE, and adoptively transferred to recipient mice. The next day, Day 0, some recipient mice were intradermally stimulated with  $1 \times 10^6$  irradiated B16N/V cells. Mice that did receive labeled CD8<sup>+</sup> T cells but were not stimulated were used as controls for the FACS analysis on Day 6.

The only two groups that showed increased proliferation of transferred CD8<sup>+</sup> T cells in stimulated versus non-stimulated mice are those purified from B16N/V and B16 $\alpha$ Gal + B16N/V treatment groups. See Figure 23A.

Among groups, transferred CD8<sup>+</sup> T cells purified from all treatment groups showed enhanced proliferation in spleens of stimulated mice when compared to CD8<sup>+</sup> T cells purified from non-vaccinated mice (Figure 23B). CD8<sup>+</sup> T cells purified from B16 $\alpha$ Gal + B16N/V-vaccinated mice showed more proliferation in spleens of stimulated and non-stimulated recipient mice than CD8<sup>+</sup> T cells purified from B16N/V-vaccinated mice. In addition, CD8<sup>+</sup> T cells purified from B16 $\alpha$ Gal + B16N/V showed more proliferation in spleens of stimulated mice than CD8<sup>+</sup> T cells purified from B16 $\alpha$ Gal + B16IL-15-vaccinated mice (Figure 23B).



**Figure 23. *In vivo* proliferation of CFSE-labeled T cells.**

CD8<sup>+</sup> T cells were purified from mice vaccinated with  $1 \times 10^6$  B16N/V cells,  $5 \times 10^5$  B16aGal +  $5 \times 10^5$  B16N/V cells or  $5 \times 10^5$  B16aGal +  $5 \times 10^5$  B16IL-15 cells, and from non-vaccinated mice. Purified CD8<sup>+</sup> T cells were then labeled with CFSE and intravenously transferred to recipient mice. The next day, Day 0, recipient mice were either stimulated or not with irradiated B16N/V melanoma cells. Lymphocytes were isolated on Day 6 and analyzed by FACS. A. Comparison of transferred CFSE-labeled CD8<sup>+</sup> T cells proliferation between stimulated versus not stimulated recipient mice (St = Stimulation). B. Comparison of transferred CFSE-labeled CD8<sup>+</sup> T cells proliferation between the groups of mice from which they were purified. Cells were gated according to their CFSE intensity. G1, G2, G3, and G4 represent cells with no, one, two or more cell divisions *in vivo*, respectively.

## DISCUSSION

An ultimate goal of a vaccine is to elicit a long-term immunological protection that can reduce the severity of a disease. Memory T and B cells and long-lived effector B cells (plasma cells) constitute the basis of this immunological memory (100) and since tumor destruction is mainly mediated by CD8<sup>+</sup> T cells, it was rational to hypothesize that combining IL-15 with the conventional B16 $\alpha$ Gal vaccine would increase the efficacy of the vaccine by enhancing the maintenance and proliferation of memory CD8<sup>+</sup> T cells against melanoma. Combination therapy with IL-15 has been widely applied by other investigators to treat tumor in murine models (49, 70, 74, 88, 101).

Thus, the main purpose of this study was to determine whether the incorporation of IL-15 with the conventional B16 $\alpha$ Gal vaccine would increase the proliferation and expansion of cytotoxic CD8<sup>+</sup> T cells and enlarge the population of long-term memory T cells. The results obtained in this study clearly demonstrated a trend supporting the hypothesis, indicating that the combination therapy could potentially result in a better strategy to treat tumors than a single vaccine regimen.

In this study several strategies were attempted to demonstrate if the combination therapy consisting of B16 $\alpha$ Gal plus B16IL-15 would result in the improvement of the treatment of melanoma tumors. Two tumor models were used. One of the models reflected the treatment of a single subcutaneous localized tumor; the other model represented the treatment of multiple disseminated metastatic pulmonary tumors. In addition, for each of these tumor models two vaccination modalities were performed, the treatment of pre-existing tumors in which animals received tumor inoculation and were



subsequently vaccinated. The other therapeutic scheme was the preventive vaccination model in which animals were vaccinated and subsequently challenged with tumor. Results from those experiments demonstrated a statistically significant improvement in the treatment and prevention of melanoma when B16 $\alpha$ Gal plus B16IL-15 combination therapy was used compared to the utilization of B16 $\alpha$ Gal (or B16 $\alpha$ Gal + B16N/V) vaccine alone in three out of 5 experiments (Figures 8, 9, and 10). Moreover, in the pre-existing pulmonary experiment although there was no significant difference in tumor burden between mice receiving the combination therapy and mice receiving B16 $\alpha$ Gal only, the same trend was observed (Figure 7 and Table 6). All together these data provide preliminary support to the hypothesis that combination therapy with IL-15 increased the efficacy of B16 $\alpha$ Gal vaccine for the treatment of melanoma

One of the possible mechanisms by which IL-15 could have potentially increased the efficacy of B16 $\alpha$ Gal vaccine was by the expansion of melanoma specific CD8<sup>+</sup> T cells and the generation of a larger pool of memory CD8<sup>+</sup> T cells that was probably able to better clear tumors upon re-encountering melanoma antigens (49, 56, 88). We asked the question whether adding B16IL-15 vaccine to the system would indeed increase the pool of long term memory CD8<sup>+</sup> T cells compared to memory cells induced by the conventional B16 $\alpha$ Gal vaccine. To answer this question CD8<sup>+</sup> T cell proliferation studies were conducted. CD8<sup>+</sup> T cell-donor mice were vaccinated and rested for several months. After this period of time, CD8<sup>+</sup> T cells were collected, labeled with CFSE, and transferred to recipient syngeneic mice that were subsequently vaccinated with irradiated B16 melanoma cells to stimulate T cell proliferation. Transferred cells were collected and proliferation was evaluated by the dilution of CFSE intensity. Results demonstrated that

CD8<sup>+</sup> T cells isolated more than 3 months after vaccination with B16 $\alpha$ Gal + B16IL-15 had indeed shown more proliferation in spleens, non-draining, and draining lymph nodes of stimulated recipient mice than CD8<sup>+</sup> T cells isolated from mice vaccinated with B16 $\alpha$ Gal + B16N/V cells (Figure 22). Thus, the combination therapy with B16 $\alpha$ Gal + B16IL-15 has demonstrated a slight overall benefit compared to the administration of B16 $\alpha$ Gal vaccine alone through out this study. Although further investigation is clearly needed to confirm these preliminary results, the benefit observed might be due to the enhanced maintenance and/or proliferation of CD8<sup>+</sup> T cells by IL-15.

One of the difficulties encountered during the course of this study was the relative increase in the efficacy of the B16N/V cell vaccines compared to the B16 $\alpha$ Gal vaccine. Previous publications by our laboratory and others had demonstrated significant protection from subcutaneous and pulmonary melanoma of mice vaccinated with  $\alpha$ Gal-expressing B16 cells compared to mice vaccinated with B16 cells that do not express the  $\alpha$ Gal epitope (43, 102, 103). The difference in the immunogenicity obtained might be explained by the difference in the cell line clones used in the previous publications and this study. The B16 cell line clone used in previous publications was the B16BL6 clone, whereas the B16F0 clone, purchased from ATTC, was used in this study. It is possible that the clone B16BL6 is less immunogenic than the clone B16F0. At this point this hypothesis was not systematically addressed and remains speculative.

In an attempt to solve this problem and be able to detect any differences between B16 $\alpha$ Gal-vaccinated mice and B16N/V-vaccinated mice, the vaccine dose was reduced by one-half, to a sub-optimal concentration. Also the vaccination site was changed. We tried targeting two lymph node groups rather than one by injecting the mice into the

midline rather than into the right flank. In spite of these efforts, only in one out of three experiments, was it possible to detect a significant difference between the two treatments (Figure 10). In that experiment, mice vaccinated with B16 $\alpha$ Gal + B16N/V had significantly less tumor metastases than mice receiving only B16N/V vaccine cells as previously reported. Currently, the utilization of an allogeneic vaccine is (see below) addressing this issue better since more consistent results are being demonstrated thus far (appendix).

Another problem encountered in this study was the relatively lower tumor take of female mice compared to male mice. As mentioned in the Results section, in two experiments (Figures 10 and 16) female mice did not develop tumor metastases as expected upon tumor implantation. This incident had been observed by other investigators in the laboratory (personal communication). One explanation would be that the B16N/V cells used in this study were derived from male mice and males express the male-specific minor histocompatibility antigen H-Y. This protein is encoded on the Y chromosome and female T cells have been shown to respond to peptides that are derived from this protein (104). That resulted in using around half the number of mice originally designated for those experiments reducing the power of analysis to detect possible smaller differences.

In this study we evaluated the induction of long-term memory cytotoxic CD8<sup>+</sup> T cells by the combination therapy by conducting adoptive T cell transfer experiments. Our results do not support that the addition of B16IL-15 vaccine to the conventional B16 $\alpha$ Gal vaccine significantly improved the induction of long-term memory CD8<sup>+</sup> T cells. In the first transfer experiment, more stringency was added to the system by lowering the

vaccine dose and injecting double the tumor cells. A significant difference was only detected between mice receiving no CD8<sup>+</sup> T cells and mice receiving CD8<sup>+</sup> T cells from mice vaccinated with B16 $\alpha$ Gal + B16IL-15. In the second transfer experiment, CD8<sup>+</sup> T cell purification technique was optimized and thus we obtained highly viable CD8<sup>+</sup> T cells, but the experiment was prematurely terminated and mice in all treated groups contained only very small tumor burdens (Figure 15). The third transfer experiment was conducted with no known problems and demonstrated that no significant differences existed between the two treatments, CD8<sup>+</sup> T cells purified from B16 $\alpha$ Gal + B16N/V-vaccinated mice and CD8<sup>+</sup> T cells purified from B16 $\alpha$ Gal + B16IL-15-vaccinated mice.

This result could be explained on the basis that B16 $\alpha$ Gal vaccine itself is very potent and induces a very strong long-lasting cytotoxic-CD8<sup>+</sup> T cell activity. It was previously demonstrated that B16 $\alpha$ Gal-vaccinated mice induced cytotoxic CD8<sup>+</sup> T cells able to treat pre-existing tumors upon transfer to syngeneic mice (43). In those experiments, adoptive T cell transfer was performed 2 to 4 weeks after vaccination. It was not clear at the initiation of this study for how long this immunologic memory would last. Experiments reported here showed that vaccination with B16 $\alpha$ Gal induced CD8<sup>+</sup> T cells able to treat most animals upon transfer (Figures 15 and 16.). The cytotoxic activity of these CD8<sup>+</sup> T cells was found several months (5 and 7 months) after vaccination. The addition of B16IL-15 in the described vaccination regimen did not show improvement in the activity of transferred cells in the time frame evaluated in this study. Consequently under these conditions, combination therapy with B16IL-15 did not induce more effective long-term memory pool of CD8<sup>+</sup> T cells compared to the conventional B16 $\alpha$ Gal vaccine.

The proliferative potential of purified CD8<sup>+</sup> T cells from vaccinated mice was also evaluated. CD8<sup>+</sup> T cells purified from mice vaccinated with B16 $\alpha$ Gal and B16IL-15 more than three months after vaccination showed more proliferation *in vivo* by CFSE (Figure 22) than CD8<sup>+</sup> T cells purified from mice vaccinated with B16 $\alpha$ Gal and B16N/V cells. This difference was not observed when CD8<sup>+</sup> T cells were isolated two weeks after the last vaccination from B16 $\alpha$ Gal + B16IL-15 vaccinated mice (Figure 23). This is consistent with prior data suggesting that IL-15 enhances the maintenance of memory CD8<sup>+</sup> T either by stimulating their proliferation or sustaining their survival (49, 56, 88).

Although outside the scope of this study, an additional possible mechanism by which IL-15 might increase the efficacy of B16 $\alpha$ Gal vaccine is through the activation of NK cells. IL-15 plays a pivotal role in the proliferation and differentiation of NK cells, another set of cytotoxic cells that kill cancer cells (49). In fact, in addition to reduction in memory CD8<sup>+</sup> T cells, IL-15<sup>-/-</sup> as well as IL-15R $\alpha$ <sup>-/-</sup> mice have significantly reduced NK cells, NKT cells, and intestinal intraepithelial lymphocytes (61). Activation and proliferation of NK cells might lead to more effective elimination of tumors in vaccinated mice. In addition, the role of IL-15 in the inhibition of IL-2-mediated activation induced cell death (AICD) is clearly established (79). This process leads to the elimination of self-reactive T cells. Combining IL-15 with B16 $\alpha$ Gal vaccine might also result in the inhibition of AICD and thus maintaining the number of self-reactive T cells that can act against tumor cells. These possible mechanisms were not evaluated in the current study, but are currently under investigation by our group.

Several techniques were optimized and mastered during the course of this study. One of the major technical contributions was the purification, CFSE labeling, and *in vivo*

tracking of CD8<sup>+</sup> T cells. The CD8<sup>+</sup> T cell purification technique was optimized. Additionally, the expected yield of viable CD8<sup>+</sup> T cells from mice was possible to recover when the technique described in this report was followed. CFSE dye is highly toxic to cells and thus it is critical to use the appropriate cell and CFSE concentrations with an effective reagent to stop the reaction. The incubation time is another important factor in this technique and in our hands the optimum incubation time was five minutes at room temperature with continuous mixing. CFSE-labeling of lymphocytes was not previously performed in our laboratory. The method described in this report allowed for efficient labeling and tracking of lymphocyte proliferation including CD8<sup>+</sup> T cells by CFSE *in vitro* and *in vivo* (Figures 17-19 and 21-23).

While conducting this project, investigators in the laboratory developed a model system that utilizes allogeneic vaccines for the treatment of melanoma, see appendix.

Many limitations are overcome by the utilization of allogeneic vaccines. One important limitation is the practical difficulty of manufacturing enough autologous cells from each individual in case of a syngeneic cancer vaccine, therefore allogeneic vaccines are clinically more feasible to prepare. In addition, evidence had shown that antigen-presenting cells are able to present tumor antigens to naïve T cells through cross-presentation and possibly inducing a cytotoxic response (105), thereby eliminating the need for MHC matching between the vaccine cells and host's cells. Allogeneic vaccines can share tumor associated antigens with autologous tumor. Studies have also shown that human melanoma tumor antigens are shared in at least 50% of patients (106). Another advantage of using allogeneic vaccines is the possibility of breaking tolerance to autologous tumor antigens which most of them are presented by normal cells (107). A

wide body of evidence supports the notion that a pre-existing state of tolerance exists against a self-antigen present in tumor cells. This tolerance might potentially be broken by presenting mutated antigens, antigens in different conformations or antigens with different post-translational modifications (108). Recent studies have shown that genetically modified and unmodified allogeneic whole cell cancer vaccines are showing anti-tumor activity and survival benefits in clinical trials (50, 109, 110). These data also encouraged our team to construct an allogeneic vaccine expressing the  $\alpha$ Gal epitope to treat pre-existing tumors in  $\alpha$ GT KO mice (appendix).

Briefly,  $\alpha$ GT KO mice (H-2<sup>b/b</sup>) bearing subcutaneous and pulmonary B16 melanoma (H-2<sup>b/b</sup>) showed enhanced survival and reduced pulmonary metastases when vaccinated with S91M3 $\alpha$ Gal (H-2<sup>d/d</sup>) allogeneic whole cell vaccine compared to mice vaccinated with S91M3 vaccine cells and non-vaccinated mice. Vaccination with S91M3 $\alpha$ Gal had also induced cytotoxic CD8<sup>+</sup> T cell activity against syngeneic B16 melanoma tumor which was measured by adoptive transfer to recipient mice bearing pulmonary metastases. Furthermore, T cells harvested from mice vaccinated with S91M3 $\alpha$ Gal were cultured with autologous antigen-presenting cells in the presence and absence of the melanoma peptide mTRP-2. The recognition of the melanoma peptide by those T cells was measured by the expression of TNF- $\alpha$  and CD3 molecule by T cells. In this model, the presence of alloantigens did not dominate the immune response and thus did not prevent the development of anti-tumor response; instead they contributed to the generation of a more effective vaccine compared to the classic autologous B16 $\alpha$ Gal vaccine. Toxicology studies revealed no signs of toxicity or autoimmunity in long-term

murine experiments using breast, lung, and melanoma models upon the administration of allogeneic vaccines.

The study shown in the appendix has been submitted for publication. I am one of the co-authors of the paper and my responsibility was mainly performing the *in vitro* T cell assays. This included collecting spleens and macrophages from mice and purifying splenocytes using the technique described in this report. Spleen mononuclear (Spm) cells were then cultured with the freshly isolated peritoneal macrophages to determine intracellular cytokines in presence or absence of peptides. The peptides used were the melanoma peptide mTRP-2 and non-melanoma peptide OVA. Intracellular TNF- $\alpha$  was detected using a mouse detection kit (BD Pharmingen) following manufacturer's instructions. Vaccination with the allogeneic S91M3 $\alpha$ Gal vaccine had induced T cells that recognized the melanoma peptide mTRP-2 presented in the context of autologous MHC. This expression was not observed when T cells were pulsed with OVA nor by T cells purified from non-vaccinated mice. In addition, the responsibilities included testing culture supernatants for IFN- $\gamma$  production by ELISA (data not shown). Finally, I participated in reviewing and editing the paper before submission.



## CONCLUSION

The combination of IL-15 with B16 $\alpha$ Gal vaccine appeared to improve the efficacy of the B16 $\alpha$ Gal vaccine in preventing and treating melanoma. This was demonstrated in four out five *in vivo* experiments using  $\alpha$ GT KO mice. In three of these experiments, the efficacy of the B16 $\alpha$ Gal vaccine was significantly enhanced when combined with B16IL-15 vaccine cells. This significance was achieved in the therapeutic subcutaneous model as well as in the preventive subcutaneous model.

CFSE labeling experiments demonstrated that CD8<sup>+</sup> T cells induced more than 3 months after vaccination with B16 $\alpha$ Gal plus B16IL-15 proliferated more than CD8<sup>+</sup> T cells induced after vaccination with B16 $\alpha$ Gal plus B16N/V. These preliminary results are consistent with prior data suggesting that IL-15 enhances the proliferation of long term memory CD8<sup>+</sup> T cells.

Unfortunately, it was not possible to reproduce some of the results shown in this report. In other experiments, for example the adoptive transfer experiments, we were not able to show significant differences between the administration of B16 $\alpha$ Gal vaccine and the combination therapy. Further characterization of long-term memory CD8<sup>+</sup> T cells using different parameters than those used in this project might highlight more differences in the quality and quantity of these cells induced by single versus combinatory treatment. Additionally, increasing the number of mice per group to increase the power of analysis to detect small differences and/or changing to the allogeneic model might confirm possible differences between the combination therapy and B16 $\alpha$ Gal vaccine therapy.

To conclude, this study showed a trend suggesting that the combination of B16IL-15 with the B16 $\alpha$ Gal vaccine might result in an improved therapeutic approach to treat melanoma in murine models.

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## **APPENDIX**

### **Allogeneic melanoma vaccine expressing $\alpha$ Gal epitopes induces anti-tumor immunity to autologous antigens without signs of toxicity**

**Paper in revision by Journal of Immunotherapy**

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#### ***Keywords***

$\alpha$ Gal epitope, melanoma, xenotransplant, adjuvant.

#### ***Footnotes***

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## Abstract

Due to the absence of  $\alpha$ Gal epitopes in human cells and constant stimulation of the immune system by the symbiotic bacterial flora, humans develop high titers of natural antibodies against these epitopes. It has been demonstrated that syngeneic whole cell vaccines modified to express  $\alpha$ Gal epitopes could be used to generate a potent anti-cancer vaccine. In this study we tested whether allogeneic whole cell cancer vaccines modified to express  $\alpha$ Gal epitopes would be effective for the treatment of murine melanoma.  $\alpha$  (1,3)Galactosyltransferase ( $\alpha$ GT) knockout mice (H-2<sup>b/b</sup>) with pre-existing subcutaneous and pulmonary tumors ( $\alpha$ Gal<sup>(-)</sup> B16, H-2<sup>b/b</sup>) received therapeutic vaccinations with S91M3 $\alpha$ Gal<sup>(+)</sup> (H-2<sup>d/d</sup>) whole cell allogeneic vaccines. These mice had better survival and reduced pulmonary metastasis burden compared to control mice treated with S91M3 vaccine cells. Vaccination with S91M3 $\alpha$ Gal induced cytotoxic CD8<sup>+</sup> T cells recognizing the syngeneic  $\alpha$ Gal<sup>(-)</sup> B16 tumors measured by adoptive transfer to recipients bearing pulmonary metastases. The presence of allo-antigens does not dominate the induction of immunity to “cryptic” tumor antigens and helped in the generation of a more efficient vaccine to treat pre-existing tumors when compared to classic autologous vaccines. Vaccination with allogeneic  $\alpha$ Gal<sup>(+)</sup> vaccines did not induce signs of toxicity including changes in weight, hematology, chemistry and histopathology of major perfused organs or autoimmunity in long-term murine models for breast, lung and melanoma. This study establishes the safety and efficacy data of allogeneic  $\alpha$ Gal<sup>(+)</sup> whole cell vaccines and constituted the basis for the initiation of human clinical trials to treat human malignancies.

## Introduction

The human immune system is continuously stimulated by intestinal and pulmonary bacterial flora to produce natural antibodies that recognize  $\alpha$ Gal epitopes (1). These anti- $\alpha$ Gal antibodies, which can reach up to 1% of circulating IgG, are capable of mediating a “hyperacute rejection” of tissues and cells expressing  $\alpha$ Gal epitopes. This phenomenon is mediated by the binding of anti- $\alpha$ Gal antibodies to  $\alpha$ Gal epitopes and complement activation through the classic pathway (2). In addition, non-complement fixing natural anti- $\alpha$ Gal antibody induces antibody-dependent cell cytotoxicity (ADCC) that initiates tissue damage (3, 4). We have exploited this immune mechanism to create a whole cell cancer vaccine to treat melanoma tumors. Previous studies using the  $\alpha(1,3)$ -galactosyltransferase ( $\alpha$ GT) knockout mouse model, demonstrated that syngeneic B16 melanoma vaccines genetically engineered to express  $\alpha$ Gal epitopes (B16 $\alpha$ Gal) effectively treated pre-existing subcutaneous and pulmonary  $\alpha$ Gal<sup>(-)</sup> melanoma (B16F0) tumors (5, 6).

In order to translate this type of vaccine to the clinic, the practical difficulties of raising enough autologous cells from each individual as well as of developing clinically acceptable standard procedures for vaccine manufacturing have to be overcome. The use of allogeneic  $\alpha$ Gal<sup>(+)</sup> vaccines would solve these limitations and making it clinically and industrially feasible to prepare a single vaccine containing a broad set of representative tumor associated antigens (TAA).

There is a considerable body of evidence showing that through the phenomenon of cross-presentation, tumor antigens from vaccine tumor cells are presented to naïve T cells by the host's antigen presenting cells, thereby eliminating the need for MHC matching between the vaccine and host's cells (7)

However, the approach of using allogeneic vaccines requires that tumor cells used as vaccines share some cross-reactive TAA with the autologous tumor. Most tumor cells have unique expression profiles of TAA, but in many cases these TAA are unknown or very difficult to identify or isolate from individual tumors. Studies show that human melanoma tumor antigens are shared in at least 50% of patients (8). Therefore, the use of whole cell vaccines alleviates this difficulty, as it provides a whole repertoire of TAA without the need to isolate or characterize those antigens.

Additionally, most tumors escape immune surveillance because the immune system has been tolerized to autologous TAA. A wide body of evidence supports the notion that a pre-existing state of tolerance against a self-antigen present in tumor cells can be broken by presentation of mutated antigens, antigens in different conformations or with different post-translational modifications (9). Allogeneic whole cell vaccines comprised of TAA showing allelic variation with the target tumor may be useful in breaking tolerance to those autologous TAA.

However, the induction of an effective anti-tumor immune response is anticipated to require the activation of autoreactive T cells that recognize autologous antigens present in tumors and normal tissues. Since most tumors share antigens with normal tissues, it is expected that an effective vaccination therapy may have a secondary effect on the induction of autoimmune reactions against normal tissues. This has been clearly

demonstrated in melanoma therapies in human clinical trials. (10, 11) and in mouse models (12-14). On the other hand, other animal studies have shown that vaccination protocols can generate substantial antitumor immunity with little, or no, autoimmunity (13, 15-18).

Genetically modified or unmodified allogeneic whole cell cancer vaccines are showing anti-tumor activity and survival benefits in clinical trials, thereby validating the hypothesis that immune rejection of laboratory produced human cancer cell lines can induce destruction of patient's malignancies (19-21).

In this study we determined whether allogeneic vaccines expressing  $\alpha$ Gal epitopes are as effective in the treatment of pre-existing tumors as previously described  $\alpha$ Gal<sup>(+)</sup> syngeneic vaccines (5, 6). Also, we assessed whether the vaccination with allogeneic vaccines expressing  $\alpha$ Gal epitopes induced signs of autoimmunity or other type of toxicity in murine models for vaccines to treat lungs, breast and melanoma cancers.



## Materials and Methods

### *$\alpha(1,3)$ Galactosyltransferase ( $\alpha$ GT) knockout (KO) animals*

Females and males 8 to 14 weeks old  $\alpha$ GT KO mice were used in this study. Several founders of the original colony  $\alpha$ GT KO mice were purchased from Dr. J.B. Lowe (University of Michigan) (22). The original  $\alpha$ GT KO mouse expressed both alleles H-2<sup>b</sup> and H-2<sup>d</sup> haplotypes. These animals were generated by crossing C57BL/6 x DBA/2J x 129sv mice (H-2<sup>b</sup> x H-2<sup>d</sup>). In an effort to obtain homozygous colonies, by breeding and selection, we generated two  $\alpha$ GT KO mouse colonies homozygous for both H-2<sup>b/b</sup> and H-2<sup>d/d</sup> haplotypes. Animals used in this study express H-2K<sup>b</sup> and H-2D<sup>b</sup> haplotypes and they do not express H-2D<sup>d</sup> or H-2K<sup>d</sup> (6).

Mice were immunized intraperitoneally (i.p.) with  $1 \times 10^8$  rabbit red blood cells (RRBC) twice, two or three weeks apart to increase the anti- $\alpha$ Gal antibody titers (23). The presence of elevated titers of anti- $\alpha$ GalAb was confirmed one week after the last RRBC immunization.

### *Cell lines and vaccine preparation*

The following murine melanoma cell lines were used in this study: B16F0 (originated in C57BL/6 mice, H-2<sup>b/b</sup>) and S91M3 (ATCC number CCL-53.1, H-2<sup>d/d</sup>). These cell lines do not express  $\alpha$ GT gene due to down regulation of the  $\alpha$ GT gene expression (24-26).

Melanoma cells were transduced with a lentiviral vector expressing  $\alpha$ GT gene to produce cell lines expressing  $\alpha$ Gal epitopes (B16 $\alpha$ Gal and S91M3 $\alpha$ Gal) as previously

described (27). Briefly, the  $\alpha$ GT murine gene was cloned into a self inactivating HIV-based vector under the control of the human PGK promoter (pHSPA). Viral vector supernatants were collected two days after transfection of 293T cells and used to stably transduce melanoma cells. The efficiency of the transduction and expression of  $\alpha$ Gal epitopes in cell lines used in this study were determined by IB4 lectin staining (6). Also the Lewis Lung Carcinoma (LLC ATTC number CRL-1642) and EMT-6 cells (ATTC number CRL-2755) were used as positive controls for staining. These cell lines naturally express the  $\alpha$ GT gene (*Supplementary 1*). For vaccine preparation, cells were expanded in complete Dulbecco's Modified Eagle medium (DMEM) media, harvested and irradiated with 200 Gy. Irradiated cells (vaccines) were stored frozen in appropriated aliquots. At the moment of inoculation, vaccine cells were thawed, washed and resuspended in sterile saline solution at the appropriate concentration for injection.

#### ***Detection of $\alpha$ Galepitopes by Fluorescence Activated Cell Sorting (FACS)***

To detect the expression of  $\alpha$ Gal epitopes, cells were stained as previously described (5, 6, 23). Briefly, 5  $\mu$ l of the fluorescein isothiocyanate (FITC)-labeled *Griffonia simplicifolia* isolectin B4 (IB4, Molecular Probes), was added to one million cells in Hybridoma Serum Free media (Invitrogen-Life Technologies), Cells were incubated for 30 minutes, washed and analyzed by FACS. This lectin has previously been shown to bind specifically to  $\alpha$ Gal epitopes (28).

### ***Animal treatments for efficacy studies***

S91M3 melanoma cells (H-2<sup>d/d</sup>) were transduced with pHSPA vector to produce S91M3 $\alpha$ Gal cells. The melanoma cell line B16F0 (H-2<sup>b/b</sup>) was used to establish pulmonary tumors in  $\alpha$ GT KO mouse (H-2<sup>b/b</sup>) as previously described (6). Mice received two or three RRBC injections to increase anti- $\alpha$ GalAb. Subsequently, they were challenged intravenously (i.v) or subcutaneously (s.c.) with  $5 \times 10^4$  to  $10^5$  B16F0 viable cells. At 4 to 5 days after tumor challenge, animals received either no treatment or were vaccinated subcutaneously with three weekly doses of  $1 \times 10^6$  S91M3 $\alpha$ Gal or control vaccines, S91M3 ( $\alpha$ Gal<sup>(-)</sup> vaccine).

Mice that had pulmonary metastases were humanely euthanized 28 days after receiving B16F0 cells. Pulmonary metastases were enumerated in a blinded manner or tumor loads were determined by weighing lungs obtained in block. Spleens were also collected for additional analysis. Mice that had pre-existing subcutaneous tumors were euthanized when their tumors reached a volume higher than 1000 mm<sup>3</sup>. To ensure minimal distress, pain or discomfort, all animal procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) approved protocols.

### ***In vitro T cell assays***

Mice were pre-immunized with RRBC and subsequently vaccinated with three s.c. injections of irradiated S91M3 $\alpha$ Gal cells one week apart. Spleen mononuclear (Spm) cells were harvested and cultured with freshly isolated peritoneal macrophages to determine intracellular cytokines in presence or absence of peptides. Intracellular TNF- $\alpha$

was detected using a mouse detection kit (BD Pharmingen) following manufacturer's instructions. Briefly,  $1 \times 10^5$  antigen-presenting cells (APCs) /mL were pulsed with mTRP-2 180-188 (SVYDFFVWL) or OVA 257-264 (SIINFEKL) peptides for two hours. Effector Spm cells were added and incubated for 6 hours in presence of Golgi Plug™ (BD, Pharmingen). Cells were harvested and stained for intracellular TNF- $\alpha$  and CD3 expression. Acquisition was performed using Coulter flow cytometer Epics Ultra™ (Miami, Florida) using Expo32 Software.

***Adoptive transfer of CD8+ T cells with anti-tumor activity***

$\alpha$ GT KO mice were intravenously injected with  $3 \times 10^5$  B16F0 viable cells and randomized 8 days before receiving adoptive T cell transfer therapy. Donor  $\alpha$ GT KO mice were first primed with two RRBC immunizations and then received three weekly vaccinations with  $1 \times 10^6$  allogeneic melanoma vaccines expressing or not  $\alpha$ Gal epitopes (S91M3 $\alpha$ Galor S91M3 vaccine cells). Two weeks after the last vaccination splenocytes were harvested and CD8+ T cells were purified by magnetic sorting as previously described (5). Recipient mice received  $2 \times 10^5$  purified CD8+ T cells administered intravenously. Pulmonary melanoma burden was determined 28 days after tumor inoculation.

### ***Dominance of the immune response.***

We used the pre-existing pulmonary melanoma metastasis model to determine if the allogeneic property of the vaccines used in this model induces an allogeneic response that dominates over the induction of anti-tumor immunity.

$\alpha$ GT KO mice were immunized as before with RRBC. One week after the last RRBC injection they were injected intravenously with  $5 \times 10^4$  viable B16 melanoma cells. On days 4, 11 and 18 after tumor inoculation mice received a total of one million vaccine cells. Animals were divided into the following groups: Group 1 received the syngeneic B16 $\alpha$ Galvaccine alone ( $10^6$  cells); Group 2 received a combination of the syngeneic B16 $\alpha$ Galvaccine ( $5 \times 10^5$  cells) with the allogeneic S91M3 $\alpha$ Galmelanoma vaccine ( $5 \times 10^5$  cells); Group 3 received an  $\alpha$ Gal<sup>(+)</sup> non-melanoma allogeneic whole cell vaccine (EMT-6 (H2-K<sup>d/d</sup> and H2-D<sup>d/d</sup>) breast cell line,  $5 \times 10^5$  cells); Group 4 received a mixture of the syngeneic B16 $\alpha$ Galvaccine ( $5 \times 10^5$  cells) and the EMT6 non-melanoma vaccine ( $5 \times 10^5$  cells); and Group 5 received no vaccination (controls).

The breast cell line EMT-6 was selected because it was shown before to share several cancer-testis antigens with melanoma (29, 30). As before, four weeks after tumor inoculation animals were euthanized and lungs metastases quantified by counting tumors in the lungs and by measuring lungs weights.

### ***Kinetics of anti- $\alpha$ Galantibody titers after administration of allogeneic vaccines***

Three groups of  $\alpha$ GT KO mice (H-2<sup>d/d</sup> haplotype) were used to evaluate the anti- $\alpha$ Galantibody response to RRBC or RRBC plus allogeneic  $\alpha$ Gal<sup>(+)</sup> cell vaccination. Group 1: were naïve  $\alpha$ GT KO mice that did not receive any immunizations. Group 2: were  $\alpha$ GT KO mice that received three intraperitoneal doses of  $10^8$  RRBC immunizations at days 1, 14 and 28. Group 3 were  $\alpha$ GT KO mice that received the same three doses of RRBC at days 1, 14 and 28 plus 6 subcutaneous doses of  $10^6$  irradiated  $\alpha$ Gal<sup>(+)</sup> LLC cells (H-2<sup>b/b</sup>) at days 35, 42, 49, 56, 63 and 70. Blood was collected from all animals at days 32 (4 days after the last RRBC immunization), 46 (4 days after the second LLC immunization) and 75 (5 days after the last LLC vaccination). The anti- $\alpha$ GalAb titers were determined by ELISA as previously described. To quantify the amount of anti- $\alpha$ GalAb (IgG or IgM) an affinity purified chicken anti- $\alpha$ GalAb standard was used. Results are expressed as Units of anti- $\alpha$ GalAb, each unit corresponding to 1  $\mu$ g/mL of anti- $\alpha$ GalAb standard.

### ***Toxicology studies in mouse models***

#### ***Allogeneic lung cancer model***

LLC cells are generated from wild type C576Bl mice (H-2<sup>b/b</sup> haplotype) and naturally express the  $\alpha$ GT gene (6). These cells were used for toxicology studies as a prototype cell for vaccination of H-2<sup>d/d</sup>  $\alpha$ GT KO mice with allogeneic cells. Females and

males were injected intraperitoneally with RRBC to increase the anti- $\alpha$ GalAb titers. One week after the last RRBC injection, they received 6 weekly doses of  $10^6$  irradiated LLC vaccine cells, s.c. Mice were weighed weekly during the course of LLC vaccination and subsequently weighed bi-weekly. Complete blood counts, differential counts and histopathology data were collected from all groups 24 hours, 2 months and 6 months after the last LLC vaccine administration.

### ***Allogeneic breast cancer vaccine model***

The EMT-6 breast tumor cell line was originated in Balb/c mice (H-2<sup>d/d</sup> haplotype) and naturally expresses the murine  $\alpha$ GT gene. These cells were used for toxicology studies for vaccination of H-2<sup>b/b</sup>  $\alpha$ GT KO mice with allogeneic cells. Female  $\alpha$ GT KO mice (H-2<sup>b/b</sup> haplotype) were vaccinated intraperitoneally twice two weeks apart with RRBC to increase titers of anti- $\alpha$ Gal antibodies. One week after the last RRBC immunization, mice received the first dose of  $5 \times 10^5$  irradiated EMT-6 ( $\alpha$ Gal<sup>(+)</sup>) cells. This dose was repeated 3 times, one week apart. Animals received a total of four subcutaneous EMT-6 vaccine doses. One week after the last EMT-6 vaccination, some mice were euthanized and tissues and blood samples were obtained for hematology and serum chemistry (one week data). Mice were monitored for a total of six months after the study was completed. Tissues and blood samples were analyzed six months after last EMT-6 vaccine (six months data). Also, body weight was measured weekly in the first month of the study and subsequently it was measured bi-weekly until the end-of the study. Necropsy and gross pathology was performed for all animals when they were euthanized. Organs in which histopathology was performed included liver, spleen,

kidneys, mammary glands, lungs and skin.

### ***Allogeneic Melanoma vaccine model***

$\alpha$ GT KO mice (H-2<sup>d/d</sup>) received two RRBC immunizations as described above. Subsequently they received 6 weekly doses of  $10^6$  B16 $\alpha$ Gal(H-2<sup>b/b</sup>) vaccines administered subcutaneously. These mice were weighed bi-weekly during 6 months. Blood samples and major perfused organs were obtained 2 weeks and 6 months after the last melanoma vaccine for analysis.

### ***Statistical analysis***

GraphPad Prism software was used for statistical analysis. Kaplan-Meier survival analysis and log-rank tests were used for curve comparisons. One-way ANOVA and or Student *t* test were used when appropriate.



## Results

### *Treatment of pre-existing pulmonary melanoma metastasis with allogeneic melanoma vaccines*

We developed a model of allogeneic vaccines to treat pre-existing metastatic melanoma using  $\alpha$ GT KO H-2<sup>b/b</sup> mice challenged with B16F0 (H-2<sup>b/b</sup>) melanoma tumor cells and vaccinated with S91M3 $\alpha$ Gal(H-2<sup>d/d</sup>) allogeneic melanoma vaccines. Allogeneic melanoma vaccines expressing  $\alpha$ Gal epitopes significantly reduced the number of pulmonary melanoma metastases (Figure 1, A). Moreover 3 out of 12 animals receiving S91M3 $\alpha$ Gal were tumor free (arrows in Supplementary Figure 1). On striking contrast vaccination with allogeneic vaccines in the absence of  $\alpha$ Gal epitopes (S91M3 vaccines) had no significant impact on the number of B16F0 tumors developed compared to animals receiving no treatment. Moreover, none of the animals in both control groups were tumor free (Supplementary Figure 1).

To confirm the data, this experiment was repeated increasing the number of animals in each group. Results confirmed that vaccination with S91M3 $\alpha$ Gal allogeneic melanoma vaccines significantly reduced the number of B16F0 metastases in lungs of this group of mice compared to animals receiving either no treatment or  $\alpha$ Gal<sup>(-)</sup> S91M3 vaccine. (Figure 1, B C D and E). Moreover, several animals in control groups had tumors localized at distal sites (peritoneal cavity and liver metastasis, Red Arrows Figure 1). None of the animals receiving S91M3 $\alpha$ Gal vaccines had disseminated disease (Figure 1 E). It is worth highlighting that animals receiving S91M3 $\alpha$ Gal vaccines had significantly smaller tumors compared to control animals (Figure 1 E).

### ***Treatment of pre-existing subcutaneous melanoma tumors***

We tested whether immunization with allogeneic S91M3 $\alpha$ Galvaccines could prolong the survival of mice bearing pre-existing subcutaneous melanoma tumors compared to vaccination with allogeneic cells lacking  $\alpha$ Gal epitopes (Figure 2).  $\alpha$ GT KO animals were primed with three RRBC immunizations to raise the titers of anti- $\alpha$ Gal antibodies and subsequently inoculated with  $10^5$  B16F0 tumor cells. Four days later they received the first dose of either saline solution,  $10^6$  S91M3 or S91M3 $\alpha$ Galvaccine cells. They received two more doses of each treatment at weekly intervals. The mean survival time for the non-vaccinated control animals was only 19 days, whereas for animals receiving S91M3 or S91M3 $\alpha$ Galvaccines was 33 and 76 days, respectively. Vaccination with allogeneic S91M3 or S91M3 $\alpha$ Gal cells produced a significant increase in survival compared to untreated controls ( $p=0.01$  and  $p=0.0001$ , respectively). The presence of  $\alpha$ Gal epitopes on the allogeneic S91M3 $\alpha$ Galvaccine cells improved the median survival (76 vs. 33 days) and long term survival proportion (45% vs. 15%) compared to immunization with S91M3 lacking  $\alpha$ Gal epitopes ( $p=0.044$ ). This experiment was repeated and similar results were found (not shown). These experiments demonstrate that vaccination of an animal having elevated titers of anti- $\alpha$ Gal antibodies with  $\alpha$ Gal<sup>(+)</sup> allogeneic tumor vaccines can effectively treat pre-existing autologous melanoma tumors improving long-term survival over allogeneic vaccination with  $\alpha$ Gal<sup>(-)</sup> cells.

***Adoptive transfer of melanoma-specific CD8<sup>+</sup> T cells induced by allogeneic vaccines expressing  $\alpha$ Gal epitopes***

We determined whether the presentation of  $\alpha$ Gal epitopes on whole cell allogeneic cancer vaccines induces T cells that recognize syngeneic melanoma cells lacking the expression of  $\alpha$ Gal epitopes. To evaluate this, we performed adoptive T-cell transfer therapy from  $\alpha$ GT KO mice that were vaccinated with S91M3 or S91M3 $\alpha$ Gal into recipients bearing syngeneic B16F0 intrapulmonary metastases (Figure 3 A and B). A significant reduction in pulmonary melanoma metastases was observed in mice receiving purified CD8<sup>+</sup> T cells from mice vaccinated with S91M3 $\alpha$ Gal compared to mice that received no treatment ( $p < 0.0001$ ), CD8<sup>+</sup> T cells from non-vaccinated animals ( $p = 0.013$ ) and CD8<sup>+</sup> T cells from mice vaccinated with S91M3 cells lacking  $\alpha$ Gal epitopes ( $p = 0.04$ ).

This result indicates that the vaccination with S91M3 $\alpha$ Gal cells induced cytotoxic CD8<sup>+</sup> T cells that recognize autologous tumor antigens. When transferred to animals with pre-existing tumors these CD8<sup>+</sup> T cells significantly reduced the lung melanoma burden when compared to un-treated and control mice.

To further demonstrate that the vaccination with  $\alpha$ Gal-expressing vaccines induces T cells recognizing autologous tumors, we cultured T cells from vaccinated animals with autologous APCs in the presence or absence of melanoma peptide. As demonstrated in Figure 3C, vaccination with allogeneic S91M3 $\alpha$ Gal induced T cells recognizing melanoma peptide mTRP-2 presented in the context of autologous MHC.

These results confirmed the induction of anti-tumor immunity directed to autologous tumor antigens.

### ***Dominance of the immune response***

Ideally, an antigenically complex vaccine is expected to elicit a broad range of specific T-cell responses to the many antigens present in the vaccine composition. However, even though the vaccine contains a plurality of potential epitopes, the T-cell response tends to become focused on just few epitopes. This economizing by the immune system is referred to as immunodominance and has hindered approaches using multivalent vaccines that deliver multiple T-cell epitopes simultaneously (31). In addition, the response to allogeneic major histocompatibility complex molecules is one of the most potent types of T cell mediated reactions that prevents allo-transplantation among non-histocompatible individuals.

In the case of whole cell cancer vaccines that express different HLA antigens than the recipient subjects, one could question the ability of such vaccine to induce anti-tumor immunity because the allogeneic response could be dominant over the T cell response elicited toward the multiplicity of different tumor antigens.

On the other hand, the non-specific activation of T cells due to the presence of allogeneic MHC antigens could improve the reactivity against “cryptic” tumor antigens.

We asked the question of whether the presence of allogeneic MHC antigens would affect the anti-tumor immune reaction induced by allogeneic vaccines in

comparison to the anti-tumor immune response previously observed towards syngeneic  $\alpha\text{Gal}^{(+)}$  whole cell vaccines (5).

We can envision three possible scenarios. First, T cells recognizing MHC alloantigens will dominate the immune response. This will prevent the development of tumor specific T cells since most tumor antigens are considered “cryptic” or non-immunodominant. Second, allo-reactivity and the induction of an efficient anti-tumor immunity could occur simultaneously and independently. Third, the immune response towards allogeneic MHC molecules could have an adjuvant effect that increases the T cell response towards tumor antigens. An allogeneic reaction towards MHC molecules could trigger the expansion of allo-reactive T cells and inflammatory effector mechanisms similar to the reactions occurring during allograft rejection. This will culminate in the activation of autologous APCs, the presentation of tumor antigens to T cells and the organization of a more effective anti-tumor immune response. These possible outcomes were tested by treatment of mice with pre-existing pulmonary melanoma tumors with different vaccine combinations (Figure 4).

As previously shown, syngeneic  $\alpha\text{Gal}^{(+)}$  vaccine cells effectively reduced the lungs melanoma metastasis burden (B16 $\alpha\text{Gal}$ alone, G1 vs. No treatment G5) (5). The combination of  $\alpha\text{Gal}^{(+)}$  syngeneic and allogeneic melanoma vaccines further improved the efficacy of the treatment compared to non treated controls (G2 vs. G5,  $p<0.0001$ ) and compared to vaccination with the syngeneic vaccine alone (G1 vs G2,  $p=0.0167$ ). Interestingly, mice vaccinated with  $\alpha\text{Gal}^{(+)}$  allogeneic breast cancer vaccine alone or in combination with syngeneic melanoma vaccine had reduced melanoma burden compared to animals receiving no treatment (G3 vs. G5,  $p=0.0053$  and G4 vs. G5,  $p<0.0001$ ). The

reduction in the lung melanoma metastasis burden in G3 and G4 might be explained by the presence of shared cancer-testis antigens in these two types of cell lines (29, 30, 32).

This experiment shows that the presence of allo-antigens in whole-cell vaccines expressing  $\alpha$ Gal epitopes does not dominate the immune response and thus preventing the development of an antitumor T cell response. Moreover, the results suggest that the presence of allogeneic MHC molecules provides an adjuvant effect helping the organization of a more efficient therapeutic anti-tumor immune response.

### ***Kinetics of anti- $\alpha$ Gal antibodies titers after administration allogeneic cell vaccines***

A question that was raised while using those animal models is whether the anti- $\alpha$ GalAb level induced by RRBC immunization remains high or is further elevated during the course of subcutaneous immunizations. This is an essential requirement for the effective induction of anti-tumor immunity using  $\alpha$ Gal<sup>(+)</sup> vaccines. To answer the question we tested the levels of anti- $\alpha$ GalAb (IgG and IgM) during a period of 46 days after the last RRBC immunization. Additionally, we determined whether the subcutaneous administration of six doses of allogeneic cells expressing  $\alpha$ Gal epitopes affected the kinetics of anti- $\alpha$ GalAb (Figure 5). As described previously, naïve  $\alpha$ GT KO mice produce low to undetectable titers of anti- $\alpha$ GalAb. All mice injected with RRBC produced more the anti- $\alpha$ GalAb compared to naïve controls. In mice receiving only RRBC the concentration of anti- $\alpha$ GalAb IgG slowly decreased over time in the 46 days study (Figure 5). The anti- $\alpha$ Gal Ab concentration was reduced in mice that received only

RRBC immunizations during the time course of this experiment. On the contrary, mice receiving LLC vaccination during a period of 46 days, showed significantly higher anti- $\alpha$ GalAb IgG concentration after administration of subsequent subcutaneous doses of allogeneic vaccines. On the other hand, the anti- $\alpha$ GalIgM titers slowly decreased after the last RRBC immunization in the presence and absence of vaccination with  $\alpha$ Gal<sup>(+)</sup> allogeneic cells. With this study we conclude that during the period in which allogeneic vaccines are administered the anti- $\alpha$ GalAb level (IgG and IgM) remains high and the anti- $\alpha$ GalAb IgG titers are further increased.

### ***Long-term toxicology studies in murine model for lung, breast and melanoma cancers.***

#### ***Growth patterns of animals receiving allogeneic vaccines***

We conducted three studies in order to evaluate long-term effects on animals receiving  $\alpha$ Gal<sup>(+)</sup> allogeneic vaccines. Mice were either untreated (naïve) or received RRBC immunizations. Test groups received also LLC vaccines (model for  $\alpha$ Gal<sup>(+)</sup> lung cancer allogeneic vaccines), EMT-6 vaccines (model for  $\alpha$ Gal<sup>(+)</sup> breast cancer allogeneic vaccines) or B16 $\alpha$ Galvaccines (model for  $\alpha$ Gal<sup>(+)</sup> melanoma vaccines). Animals were monitored for six months measuring body weight as an indication of a general animal condition (*Supplementary 2*).

For all three models we conclude that the administration of allogeneic vaccines has no impact on the growth patterns of immunized animals.

***Hematology data of animals receiving allogeneic vaccines***

Blood samples from animals enrolled in these studies were obtained at different time points after the administration of allogeneic vaccines. Complete blood counts and differential counts were determined for the allogeneic lung, breast and melanoma vaccine models. No significant differences were found in groups receiving no treatment, RRBC immunizations only or RRBC immunizations plus allogeneic vaccines expressing  $\alpha\text{Gal}^{(+)}$  epitopes ( *Supplementary 3*).

***Histopathology data of animals receiving allogeneic vaccines***

Tissues were collected from mice at different points after receiving allogeneic vaccines (*Supplementary 4*).

Histological evaluation of heart, liver, kidneys, spleen, breast and skin for each mouse in all groups resulted in the majority of samples being within normal histological limits. The microscopic lesions observed in these studies are not uncommon in conventionally-reared mice and were not considered significant.



## Discussion

In the present study we found that the administration of allogeneic  $\alpha$ Gal-expressing melanoma vaccine induces reduction in the lung melanoma metastasis burden in mice with pre-existing lungs tumors and prolongation of mean survival time in mice with pre-existing subcutaneous tumors. The efficacy of the vaccine is mediated by CD8<sup>+</sup> T cells as demonstrated by adoptive T cell transfer studies. Additionally, we demonstrated that the presence of allogeneic MHC molecules in the vaccine cells does not dominate the induction of an effective antitumor response, but instead it seems to provide an enhancing antitumor effect. Finally, the results show that an effective antitumor response in this therapeutic setting does not seem to be associated with concomitant autoimmunity.

In our experiments, a significant extension in the median survival time or reduction in tumor burden can be obtained by vaccination using whole cell allogeneic cells. However, a significant increase in survival time and reduction in tumor burden can be further obtained by modifying allogeneic vaccine cells with  $\alpha$ Gal epitopes.

Our data showed that  $\alpha$ Gal<sup>(+)</sup> allogeneic melanoma cell vaccine can prolong the survival and achieve a complete response in 45% of animals bearing early stage pre-established (i.e 4-5 days) B16F0 tumors.

The mechanism proposed for the enhanced efficacy of allogeneic S91M3 $\alpha$ Galvaccine cells compared to S91M3 is based on the interaction of this vaccine with pre-existing anti- $\alpha$ Gal antibodies. Opsonization of  $\alpha$ Gal<sup>(+)</sup> vaccine cells by anti- $\alpha$ Gal antibodies would promote their phagocytosis by macrophages and other antigen

presenting cells. Additionally, opsonization would favor the uptake of immunocomplexes through Fc $\gamma$  receptors, which promotes the efficient uptake of antigen, cross-presentation in both MHC Class I and II, and APC activation and maturation of dendritic cells. Thus, antigen presentation by endogenous APCs and cross presentation make MHC matching between the vaccine tumor cells and the vaccine recipient unnecessary.

Although we do not know the possible cross-reactive antigen(s) being targeted by the immune response, adoptive T cell transfer and *in vitro* T cell analysis showed that the allogeneic vaccine generated CTLs specific for B16F0 and T cells recognizing mTRP-2 peptide. The present allogeneic vaccine model has clinical significance as all the human melanoma antigens identified so far are shared by at least 50% of patients. Indeed, other tumors like renal carcinoma appear to follow the same pattern and 35% of lung carcinomas expresses the antigen MAGE 28 (33).

In the present study we observed no evidence of toxicity or autoimmune effects. We evaluated several parameters in three animal models for allogeneic vaccines expressing  $\alpha$ Gal. We conclude that the administration of allogeneic vaccine has no impact on the growth patterns of animals when compared to animals receiving RRBC only and animals receiving no treatment. We also demonstrated that no significant abnormalities were observed in major perfused organs analyzed by macroscopic necropsy and histopathology. Mice receiving no vaccine (RRBC only), EMT-6, LLC or B16 $\alpha$ Galvaccines and non-manipulated aged and sex-matched naïve mice had similar lesions in lungs and other organs analyzed. No significant differences were observed in the hematology results including the hemogram, differential blood counts and blood chemistry results in any of the groups in all of the experiments performed. .

Consequently, we conclude that the administration of allogeneic vaccines does not induce any signs of autoimmunity or other type of toxicity. These results provide convincing evidence for the safety of  $\alpha\text{Gal}^{(+)}$  allogeneic vaccines for future clinical trials.

## **Acknowledgements**

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## Figure Legends

### **Figure 1: Treatment of pre-existing pulmonary melanoma tumors.**

Mice were immunized i.p. with RRBC to increase the anti- $\alpha$ GalAb titers. One week after the last RRBC immunization, pulmonary metastases were established by i.v. injection of  $8 \times 10^4$  viable B16F0 cells. Mice received either  $1 \times 10^6$  S91M3 or S91M3 $\alpha$ Galvaccine cells or no treatment on days 4, 12 and 19 after tumor inoculation. On day 28 after tumor inoculation, mice were humanely euthanized and lungs were collected. Pulmonary tumors were enumerated in a blinded manner (A). Results from two experiments are shown and express the mean of tumors burden in each group and errors are the SEM (A and B). The number of animals in each group is indicated. Lungs pictures from animals in panel B are shown for non-vaccinated animals (C) or from animals vaccinated with S91M3 (D) or S91M3 $\alpha$ Gal(E). Arrows show tumors localized in distal sites (peritoneal cavity and liver metastasis) in control groups. .

### **Figure 2. Treatment of pre-existing subcutaneous melanoma tumors.**

Mice were vaccinated with RRBC as before and then challenged subcutaneously with  $10^5$  B16F0. On days 5, 12 and 19 they received  $1 \times 10^6$  S91M3 or S91M3 $\alpha$ Galvaccine cells. Survival analysis was performed using log-rank test. The difference in the mean survival using log-rank test is indicated

**Figure 3 Adoptive T cell transfer of melanoma specific T cells induced by allogeneic vaccines expressing  $\alpha$ Gal.**

A. Donor mice were vaccinated with RRBC as explained before. One week after the last RRBC immunization, mice received three weekly doses of whole cell vaccines (S91M3 or S91M3 $\alpha$ Gal). Two weeks after the last vaccination mice were humanely euthanized and spleens were collected. CD8<sup>+</sup> T cells were purified by magnetic cell sorting. Recipient mice were injected i.v. with  $3 \times 10^5$  B16F0 viable cells. Eight days after tumor challenge they were randomized into four different groups and subjected to the following treatments: Group 1 received no T cells (T1); group 2 were inoculated intravenously with  $2 \times 10^5$  purified CD8<sup>+</sup>T cells from non-vaccinated animals (T2); group 3 received CD8<sup>+</sup> T cells from mice vaccinated with S91M3 cells (T3) and group 4 received CD8<sup>+</sup> T cells from mice vaccinated with S91M3 $\alpha$ Gal(T4). As a control, aged matched untreated, tumor free animals were included. Four weeks after tumor inoculation mice were euthanized and lungs were collected. Lungs weight was determined and plotted. Results express the mean lungs weight. Error bars = SEM. One-way ANOVA  $p= 0.0004$  for all data sets. One-way ANOVA excluding tumor free animals  $p=0.0045$ . **B.** Lung pictures of recipient mice in each group (excluding tumor free animals). **C.** T cells from naïve and from mice vaccinated with S91M $\alpha$ Gal were cultured in presence of autologous APC pulsed with OVA or mTRP-2 peptides. After 6 hours of incubation in presence of brefelidin A cells were harvested and the intracellular cytokine TNF- $\alpha$  was detected.

**Figure 4: Testing for immunodominance of the response induced by allogeneic vaccines expressing  $\alpha$ Gal epitopes**

Mice were immunized i.p. with RRBC to increase the anti- $\alpha$ GalAb titers. One week after last RRBC, pulmonary metastases were established by i.v. injection of  $5 \times 10^4$  viable B16F0 cells. Mice received a total of  $1 \times 10^6$  vaccine cells as indicated in Materials and Methods on days 4, 11 and 18 after tumor inoculation. On day 29, mice were humanely euthanized and lungs were collected. Pulmonary tumors were enumerated in a blinded manner. One way ANOVA  $p < 0.0001$ .

**Figure 5. Kinetics of anti- $\alpha$ GalAb after RRBC immunizations in presence or absence of  $\alpha$ Gal<sup>(+)</sup> allogeneic vaccines**

A. Schematic representation of immunizations and blood collections. Mice were immunized with RRBC three times two weeks apart. They received three doses of  $1 \times 10^8$  RRBC administered intraperitoneally. Mice receiving LLC cells were vaccinated at weekly intervals starting 7 days after the last RRBC immunization. All mice were bled at days 4, 19 and 46 after the last RRBC immunization.

B. Levels of anti- $\alpha$ GalIgG Ab determined by ELISA. Statistical comparison indicates that the anti- $\alpha$ GalIgG Ab titers from the groups of mice receiving RRBC or RRBC plus LLC cells are not different at day 4 (t test  $p = 0.35$ ). However, on days 19 and 46 both groups differed significantly indicating that the subcutaneous administration of LLC vaccines significantly increased the titers of anti- $\alpha$ GalAb IgG ( $p = 0.02$  for day 19 and  $p = 0.004$  for day 46). C. Levels of anti- $\alpha$ GalIgM Ab determined by ELISA. For anti- $\alpha$ GalIgM Ab

titers there is no difference among the groups receiving RRBC only and mice receiving the LLC test vaccine at any time point ( $p>0.05$  for days 4, 18 and 46). In all experiments the level of anti  $\alpha$ GalAb (IgG and IgM) of immunized mice differs significantly from naive mice ( $p<0.01$ ).



Figures

Figure 1

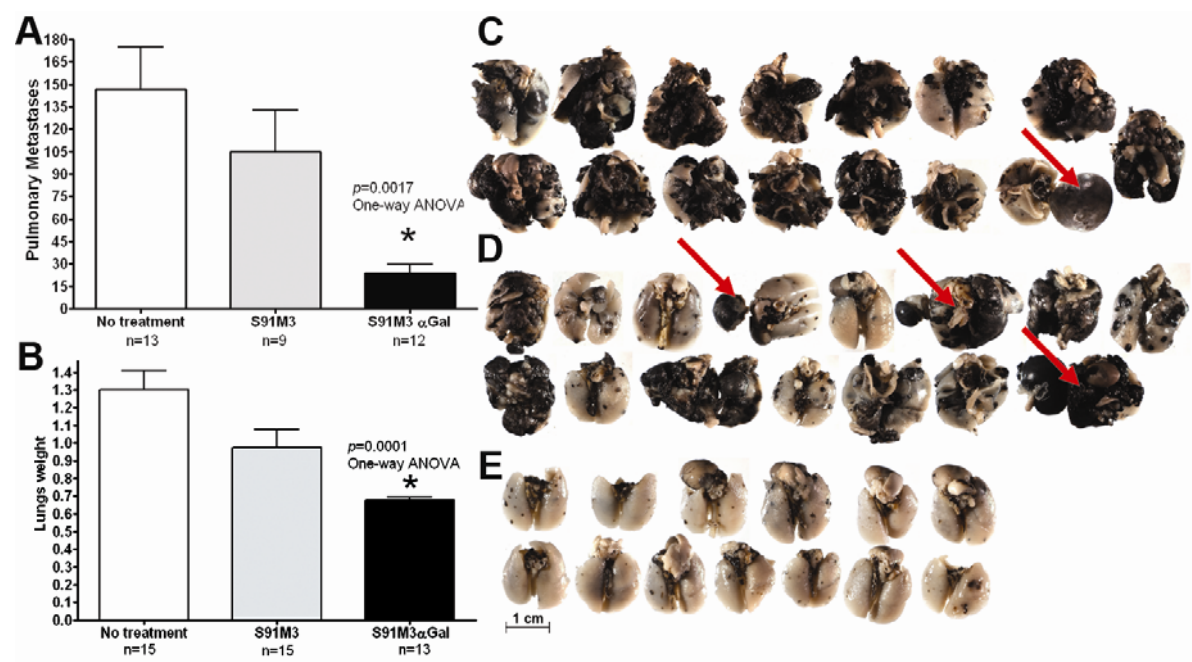


Figure 2

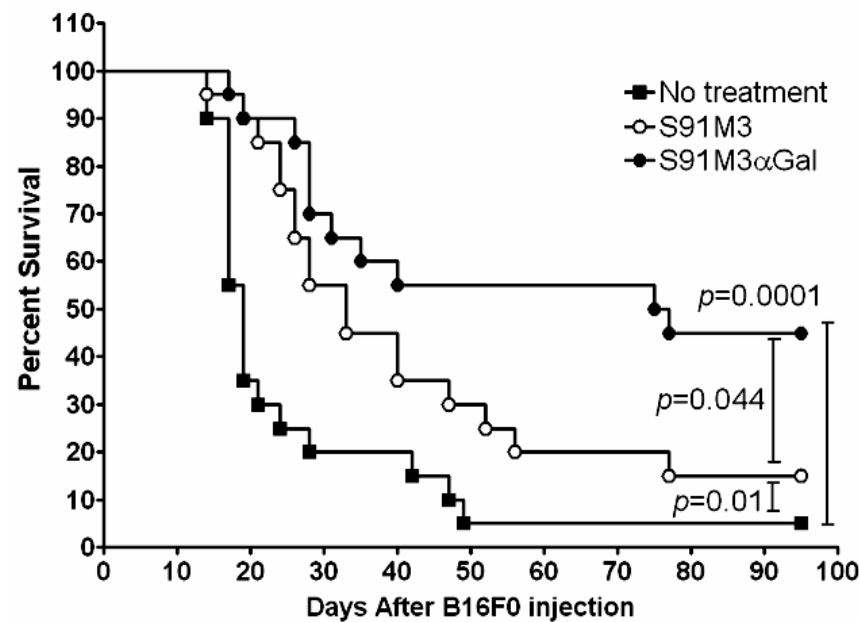


Figure 3

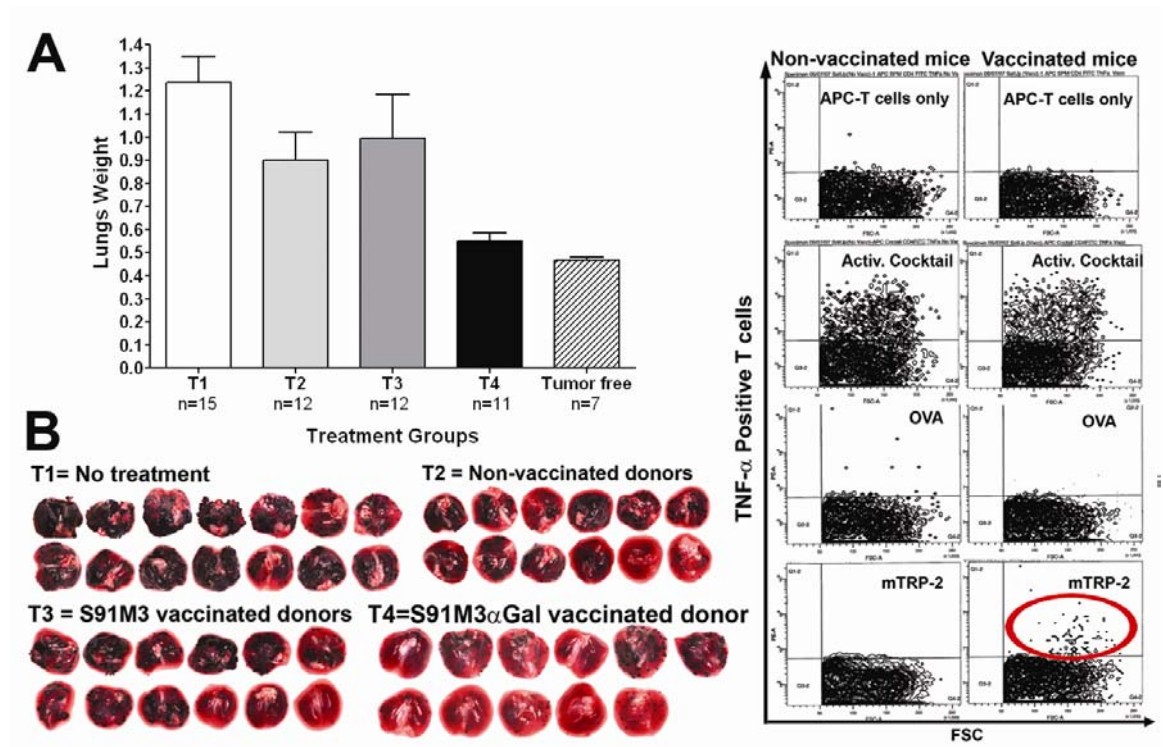


Figure 4

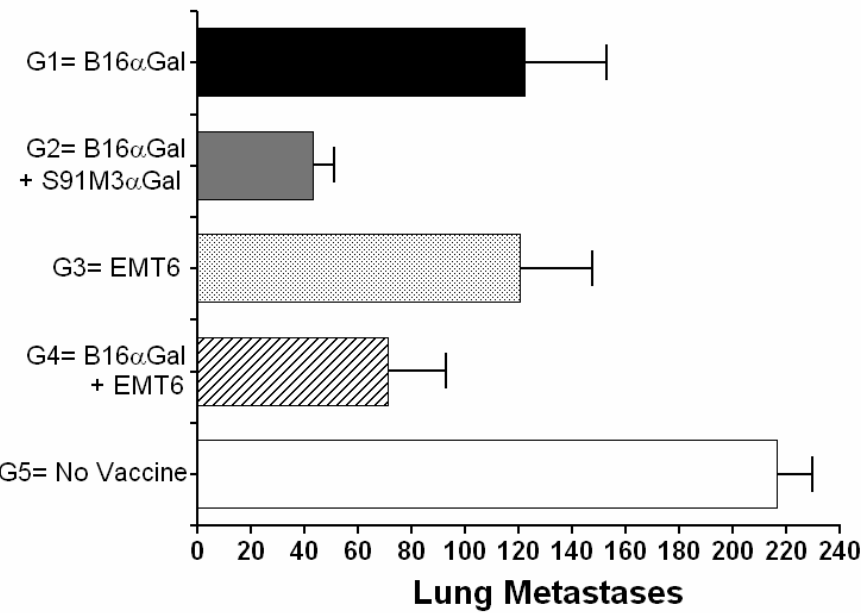
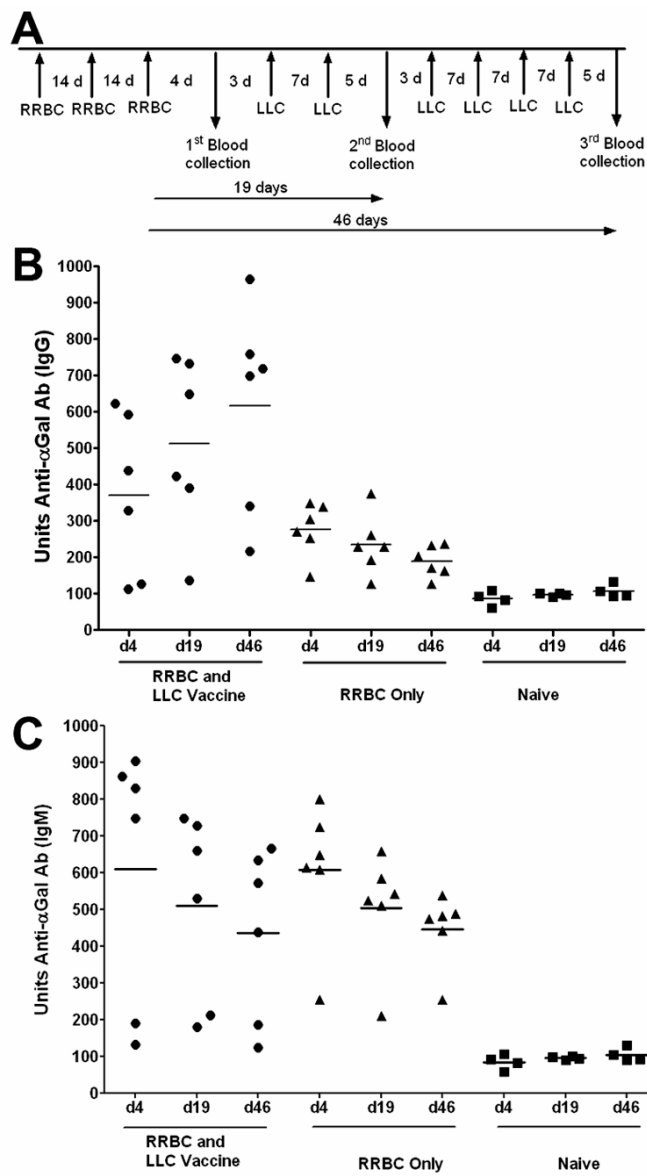


Figure 5



## Supplementary Data

Supplementary 1 Figure 1

No treatment



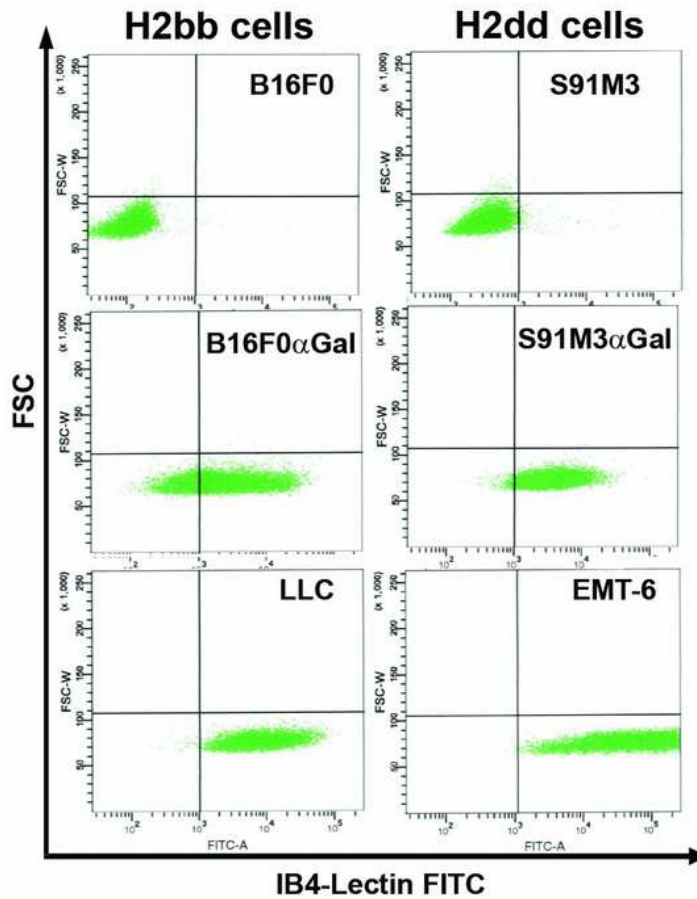
S91M3 vaccine



S91M3 $\alpha$ Gal vaccine



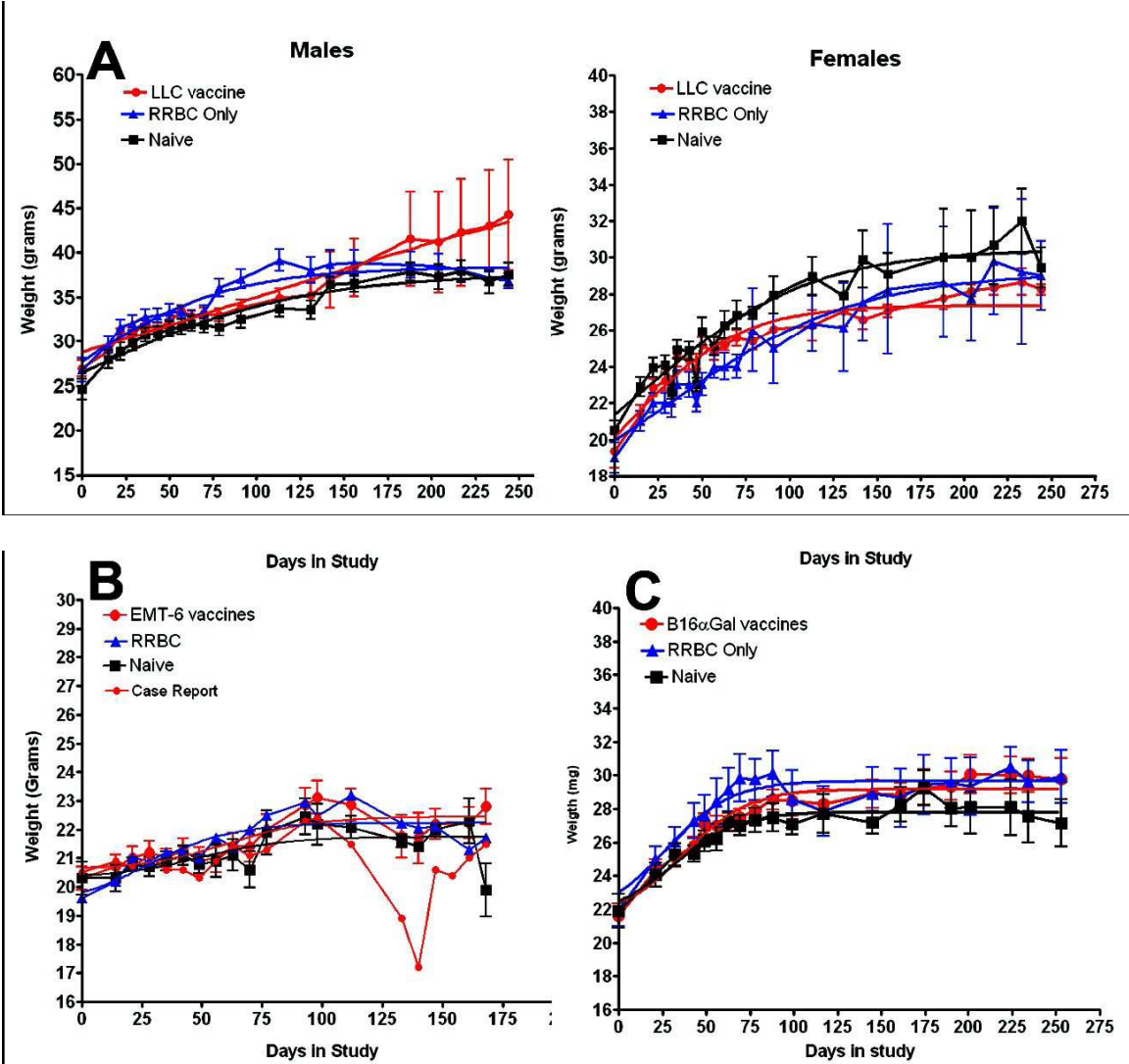
Supplementary 1 Figure 2



**Supplementary 2 Figure 1. Growth patterns of mice receiving allogeneic cancer vaccines expressing  $\alpha$ Gal epitopes.**

A. Allogeneic lung cancer vaccine model. Naive mice (males n=14, females n=15), mice injected with RRBC only (males n=10, females n=11) or injected with RRBC and vaccinated with 6 doses of LLC vaccine (males n=15, females n=14 n) were weighed for 6 months after initiation of the study. Statistical comparisons of the growth patterns indicate no significant difference among groups. The preferred model to compare the

growth patterns was the sigmoidal dose-response (variable slope). All data sets shared the same slope (males  $p=0.1654$ , females  $p=0.1822$ ). B. Allogeneic breast cancer model. Naïve mice ( $n=9$ ), mice injected with RRBC twice two weeks apart (control,  $n=13$ ) and mice receiving RRBC and EMT-6 vaccine (test group,  $n=13$ ) were monitored for six months after initiation of the study. In the study conducted for allogeneic breast cancer model one female from the test group suffered serious trauma around the nose possible due to an aggressive dominant female housed in the same cage. This animal showed significant weight lost. A different environment and enrichment was provided to this animal, which recovered 100% of its initial body weight. This case was annotated as severe and transient weight loss not related to the administration of allogeneic breast cancer vaccine. C. Allogeneic melanoma model. Naïve mice ( $n=12$ ), mice injected twice with RRBC three weeks apart (Control,  $n=9$ ) and test group (B16 $\alpha$ Gal vaccine,  $n=17$ ) receiving RRBC and six SC doses of B16 $\alpha$ Gal vaccines (1 million cells /mice/dose were monitored for six months after initiation of the study. For all data sets, the preferred model to fit the data was the sigmoidal dose-response (variable slope). All data sets shared the same slope ( $p= 0.0706$ ).



**Supplementary 3 Table 1. Hematology Results in the allogeneic vaccine for Lung Cancer Model. Data at 24 hours post LLC vaccines**

| <b>Allogeneic Lung Cancer model 24 hours after last LLC vaccine</b> |                          |      |   |                        |       |   |                                   |       |   |                          |
|---|--------------------------|------|---|------------------------|-------|---|-----------------------------------|-------|---|--------------------------|
| <b>Hemogram</b>   | <b>LLC Vaccine Group</b> |      |   | <b>RRBC only Group</b> |       |   | <b>Naïve (no treatment) group</b> |       |   | <b>ANOVA</b><br>p>0.05 * |
|   | mean                     | sd   | n | mean                   | sd    | n | mean                              | sd    | n |                          |
| WBC   | 16.6                     | 6.4  | 4 | 16.6                   | 4.0   | 4 | 16.9                              | 5.4   | 4 | ns                       |
| RBC   | 9.0                      | 1.0  | 4 | 8.5                    | 0.3   | 4 | 9.0                               | 0.6   | 4 | ns                       |
| Hemoglobin  | 14.8                     | 1.5  | 4 | 14.6                   | 0.3   | 4 | 14.8                              | 1.1   | 4 | ns                       |
| Hematocrit  | 79.5                     | 7.3  | 4 | 78.3                   | 1.6   | 4 | 80.2                              | 4.9   | 4 | ns                       |
| MCV   | 88.6                     | 5.8  | 4 | 92.5                   | 2.6   | 4 | 89.3                              | 0.7   | 4 | ns                       |
| MCH   | 16.4                     | 0.6  | 4 | 17.2                   | 0.3   | 4 | 16.5                              | 0.1   | 4 | ns                       |
| MCHC  | 18.6                     | 0.5  | 4 | 18.7                   | 0.2   | 4 | 18.5                              | 0.2   | 4 | ns                       |
| Platelet - Auto   | 953.0                    | 69.8 | 4 | 955.5                  | 105.9 | 4 | 945.0                             | 111.3 | 4 | ns                       |
| <b>Differential Counts</b>  |                          |      |   |                        |       |   |                                   |       |   |                          |
| Neutrophil  | 2.3                      | 0.6  | 4 | 2.2                    | 0.7   | 4 | 2.3                               | 1.1   | 4 | ns                       |
| Lymphocyte  | 13.0                     | 4.5  | 4 | 12.7                   | 3.3   | 4 | 13.3                              | 4.2   | 4 | ns                       |
| Monocyte  | 0.7                      | 0.6  | 3 | 0.5                    | 0.2   | 4 | 0.3                               | 0.2   | 4 | ns                       |
| Eosinophil  | 0.8                      | 1.2  | 4 | 1.3                    | 0.9   | 4 | 1.1                               | 1.2   | 4 | ns                       |
| Basophils   |                          |      | 0 |                        |       | 0 |                                   |       | 0 | ns                       |
| RDW   | 20.7                     | 0.9  | 4 | 19.7                   | 1.9   | 4 | 19.2                              | 0.5   | 4 | ns                       |
| Nucleated RBC   | 0.0                      | 0.0  | 4 | 0.0                    | 0.0   | 4 | 0.0                               | 0.0   | 4 |                          |
| <b>Chemistry</b>  |                          |      |   |                        |       |   |                                   |       |   |                          |
| BUN   | 26.8                     | 2.8  | 4 | 26.0                   | 5.2   | 6 | 22.4                              | 4.7   | 5 | ns                       |
| Creat   |                          |      | 4 | 0.0                    | 0.0   | 6 |                                   |       | 5 | ns                       |
| Glucose   | 153.8                    | 23.0 | 4 | 142.0                  | 29.4  | 6 | 130.0                             | 32.6  | 5 | ns                       |
| Total Protein   | 5.3                      | 0.6  | 4 | 5.1                    | 0.7   | 6 | 5.4                               | 0.3   | 5 | ns                       |
| Albumin   | 3.2                      | 0.3  | 4 | 3.2                    | 0.3   | 6 | 3.5                               | 0.3   | 5 | ns                       |
| Alk Phos  | 79.0                     | 6.6  | 4 | 86.0                   | 19.2  | 6 | 92.0                              | 12.6  | 5 | ns                       |
| ALT   | 49.0                     | 30.6 | 3 | 49.3                   | 39.1  | 6 | 28.4                              | 4.8   | 5 | ns                       |
| Calcium   | 9.6                      | 1.0  | 4 | 9.7                    | 0.2   | 6 | 10.0                              | 0.1   | 5 | ns                       |



**Supplementary 3 Table 2. Hematology Results Allogeneic vaccine for Lung Cancer Model. Data 2 months post LLC vaccines**

|                            | <b>Allogeneic Lung Cancer model 2 months after last LLC vaccine</b> |       |   |                    |        |   |              |
|----------------------------|---|-------|---|--------------------|--------|---|--------------|
|                            | <b>LLC Vaccine Group</b>  |       |   | <b>Naïve Group</b> |        |   | <b>ANOVA</b> |
| <b>Hemogram</b>            | mean  | sd    | n | mean               | sd     | n | p > 0.05*    |
| WBC                        | 16.82   | 8.63  | 5 | 17.53              | 5.69   | 6 | ns           |
| RBC                        | 8.76  | 0.51  | 5 | 8.48               | 0.36   | 6 | ns           |
| Hemoglobin                 | 14.28   | 0.35  | 5 | 14.25              | 0.58   | 6 | ns           |
| Hematocrit                 | 77.16   | 3.38  | 5 | 77.05              | 3.16   | 6 | ns           |
| MCV                        | 88.06   | 2.30  | 5 | 90.93              | 1.87   | 6 | ns           |
| MCH                        | 16.30   | 0.68  | 5 | 16.82              | 0.33   | 6 | ns           |
| MCHC                       | 18.52   | 0.40  | 5 | 18.48              | 0.42   | 6 | ns           |
| Platelet - Auto            | 905.40  | 67.50 | 5 | 877.17             | 106.21 | 6 | ns           |
| <b>Differential counts</b> |   |       |   |                    |        |   |              |
| Neutrophil                 | 1.53  | 0.96  | 5 | 1.01               | 1.22   | 6 | ns           |
| Lymphocyte                 | 13.23   | 7.69  | 5 | 14.02              | 4.45   | 6 | ns           |
| Monocyte                   | 0.47  | 0.30  | 5 | 0.51               | 0.25   | 4 | ns           |
| Eosinophil                 | 1.60  | 0.70  | 5 | 1.36               | 1.17   | 6 | ns           |
| RDW                        | 20.96   | 1.36  | 5 | 20.08              | 1.25   | 6 | ns           |
| Nucleated RBC              | 0.00  | 0.00  | 5 | 0.00               | 0.00   | 6 | ns           |
| <b>Chemistry</b>           |   |       |   |                    |        |   | ns           |
| BUN                        | 24.60   | 3.58  | 5 | 22.67              | 5.43   | 6 | ns           |
| Creat                      | 0.10  | 0.00  | 5 | 0.10               | 0.00   | 6 | ns           |
| Glucose                    | 126.40  | 21.80 | 5 | 116.67             | 30.60  | 6 | ns           |
| Total Protein              | 5.38  | 0.19  | 5 | 4.97               | 0.23   | 6 | ns           |
| Albumin                    | 3.22  | 0.34  | 5 | 3.07               | 0.33   | 6 | ns           |
| Alk Phos                   | 83.40   | 8.68  | 5 | 84.33              | 19.06  | 6 | ns           |
| ALT                        | 40.80   | 5.85  | 5 | 44.67              | 11.48  | 6 | ns           |
| Calcium                    | 9.96  | 0.17  | 5 | 9.48               | 0.93   | 6 | ns           |

**Supplementary 3 Table 3. Hematology Results Allogeneic vaccine for Lung Cancer Model. Data 6 months post LLC vaccines**

|                            | <b>Allogeneic Lung Cancer model 6 months after last LLC vaccine</b> |        |   |                 |        |   |             |        |   |                     |
|----------------------------|---|--------|---|-----------------|--------|---|-------------|--------|---|---------------------|
|                            | LLC vaccine group   |        |   | RRBC only group |        |   | Naïve Group |        |   | ANOVA               |
| Hemogram                   | mean  | sd     | n | mean            | sd     | n | mean        | sd     | n | p>0.05*             |
| WBC                        | 13.91   | 3.92   | 6 | 18.44           | 5.26   | 7 | 10.83       | 3.44   | 4 | 0.0418*             |
| RBC                        | 8.89  | 0.64   | 6 | 8.40            | 0.80   | 7 | 9.44        | 0.80   | 4 | ns                  |
| Hemoglobin                 | 14.80   | 1.27   | 6 | 14.00           | 0.86   | 7 | 14.20       | 1.34   | 4 | ns                  |
| Hematocrit                 | 47.87   | 4.64   | 6 | 45.34           | 2.88   | 7 | 48.23       | 3.69   | 4 | ns                  |
| MCV                        | 53.82   | 2.32   | 6 | 54.09           | 1.98   | 7 | 51.13       | 2.99   | 4 | ns                  |
| MCHC                       | 30.98   | 0.76   | 6 | 30.90           | 1.78   | 7 | 29.48       | 0.68   | 4 | ns                  |
| Platelet - Auto            | 524.33  | 310.35 | 6 | 859.29          | 250.84 | 7 | 987.25      | 319.46 | 4 | ns                  |
| <b>Differential Counts</b> |   |        |   |                 |        |   |             |        |   |                     |
| Neutrophil                 | 3.00  | 4.16   | 6 | 2.35            | 1.42   | 7 | 1.82        | 0.74   | 4 | ns                  |
| Band Neutrophil            | 0.00  | 0.00   | 6 | 0.00            | 0.00   | 7 | 0.03        | 0.05   | 4 | ns                  |
| Lymphocyte                 | 9.14  | 1.59   | 6 | 14.00           | 4.16   | 7 | 8.32        | 3.09   | 4 | 0.0181 <sup>Δ</sup> |
| Monocyte                   | 0.44  | 0.51   | 6 | 0.56            | 0.42   | 7 | 0.22        | 0.27   | 4 | ns                  |
| Eosinophil                 | 1.34  | 1.31   | 6 | 1.53            | 0.68   | 7 | 0.44        | 0.43   | 4 | ns                  |
| Basophils                  | 0.00  | 0.00   | 6 | 0.00            | 0.00   | 7 | 0.00        | 0.00   | 4 | ns                  |
| <b>Chemistry</b>           |   |        |   |                 |        |   |             |        |   |                     |
| BUN                        | 24.20   | 3.63   | 5 | 21.14           | 2.91   | 7 | 24.38       | 4.50   | 8 | ns                  |
| Creat                      | 0.2   | 0.00   | 5 | 0.2             | 0.00   | 7 | 2           | 0.00   | 8 | ns                  |
| Glucose                    | 138.40  | 39.84  | 5 | 127.29          | 7.80   | 7 | 103.63      | 29.11  | 8 | ns                  |
| Total Protein              | 5.78  | 0.54   | 5 | 5.54            | 0.24   | 7 | 5.53        | 0.45   | 8 | ns                  |
| Albumin                    | 3.28  | 0.38   | 5 | 3.26            | 0.35   | 7 | 3.24        | 0.21   | 8 | ns                  |
| Alk Phos                   | 84.60   | 17.87  | 5 | 83.43           | 9.38   | 7 | 87.00       | 21.11  | 8 | ns                  |
| ALT                        | 74.20   | 27.95  | 5 | 64.14           | 34.73  | 7 | 51.75       | 18.73  | 8 | ns                  |
| Calcium                    | 10.42   | 0.33   | 5 | 10.31           | 0.28   | 7 | 10.28       | 0.18   | 8 | ns                  |

**Supplementary 3 Table 4. Hematology Results Allogeneic vaccine for Breast Cancer Model. Data at one week post EMT-6 vaccines**

|                  | <b>Allogeneic Breast Model 1 week after last EMT-6 Vaccine</b> |       |   |                        |       |   |                    |       |   |              |
|------------------|--|-------|---|------------------------|-------|---|--------------------|-------|---|--------------|
|                  | <b>EMT-6 Vaccine group</b>                                     |       |   | <b>RRBC only group</b> |       |   | <b>Naïve group</b> |       |   | <b>ANOVA</b> |
| <b>Hemogram</b>  | Mean   | SD    | n | Mean                   | SD    | n | Mean               | SD    | n | p>0.05       |
| WBC              | 11.17  | 2.22  | 3 | 6.58                   | 0.73  | 4 | 10.70              | 5.14  | 3 | ns           |
| RBC              | 6.86   | 2.64  | 3 | 7.92                   | 0.47  | 4 | 7.26               | 0.19  | 3 | ns           |
| Hemoglobin       | 10.77  | 4.25  | 3 | 12.50                  | 0.89  | 4 | 11.30              | 0.66  | 3 | ns           |
| Hematocrit       | 35.33  | 13.84 | 3 | 40.78                  | 2.92  | 4 | 36.73              | 1.69  | 3 | ns           |
| MCV              | 51.40  | 0.44  | 3 | 51.43                  | 0.68  | 4 | 50.63              | 2.49  | 3 | ns           |
| MCH              | 15.63  | 0.55  | 3 | 15.80                  | 0.23  | 4 | 15.57              | 0.95  | 3 | ns           |
| MCHC             | 30.43  | 0.85  | 3 | 30.63                  | 0.13  | 4 | 30.77              | 0.42  | 3 | ns           |
| <b>Chemistry</b> |  |       |   |                        |       |   |                    |       |   |              |
| sodium           | 161.00   | 8.66  | 3 | 157.25                 | 2.17  | 4 | 160.00             | 1.73  | 3 | ns           |
| potassium        | 4.70   | 0.40  | 3 | 4.90                   | 0.25  | 4 | 4.77               | 0.25  | 3 | ns           |
| chloride         | 124.33   | 8.50  | 3 | 121.50                 | 1.66  | 4 | 124.00             | 2.00  | 3 | ns           |
| CO2              | 10.33  | 4.16  | 3 | 12.00                  | 2.35  | 4 | 10.00              | 1.00  | 3 | ns           |
| calcium          | 9.43   | 0.23  | 3 | 9.33                   | 0.15  | 4 | 9.23               | 0.21  | 3 | ns           |
| BUN              | 21.00  | 6.93  | 3 | 24.50                  | 8.08  | 4 | 21.00              | 2.00  | 3 | ns           |
| creatinine       | 0.20   | 0.17  | 3 | 0.20                   | 0.00  | 3 | 0.23               | 0.06  | 3 | ns           |
| Glucose          | 115.67   | 24.11 | 3 | 68.25                  | 39.42 | 4 | 55.00              | 28.93 | 3 | ns           |
| total protein    | 4.23   | 0.76  | 3 | 3.93                   | 0.54  | 4 | 4.43               | 0.12  | 3 | ns           |
| albumin          | 2.63   | 0.47  | 3 | 2.85                   | 0.05  | 2 | 2.73               | 0.15  | 3 | ns           |
| alk phosphatase  | 155.00   | 65.38 | 3 | 154.75                 | 29.45 | 4 | 189.33             | 39.40 | 3 | ns           |

**Supplementary 3 Table 5. Hematology Results Allogeneic vaccine for Breast Cancer Model. Data 6 months post EMT-6 vaccines**

| Allogeneic Breast Model 6 months after last EMT-6 Vaccine |                     |        |   |                 |        |   |             |       |   |           |
|---|---------------------|--------|---|-----------------|--------|---|-------------|-------|---|-----------|
|   | EMT-6 Vaccine group |        |   | RRBC only group |        |   | Naive group |       |   | ANOVA     |
| Hemogram  | Mean                | SD     | n | Mean            | SD     | n | Mean        | SD    | n | p>0.05    |
| WBC   | 9.66                | 2.73   | 5 | 12.43           | 4.09   | 6 | 13.20       | 2.69  | 3 | ns        |
| RBC   | 7.91                | 1.29   | 5 | 8.87            | 0.92   | 6 | 9.13        | 1.07  | 3 | ns        |
| Hemoglobin  | 12.44               | 1.77   | 5 | 13.85           | 1.27   | 6 | 13.95       | 1.48  | 3 | ns        |
| Hematocrit  | 41.62               | 5.88   | 5 | 46.07           | 4.57   | 6 | 46.65       | 4.88  | 3 | ns        |
| MCV   | 50.86               | 4.86   | 5 | 51.93           | 1.01   | 6 | 51.15       | 0.64  | 3 | ns        |
| MCH   | 15.80               | 0.70   | 5 | 15.65           | 0.65   | 6 | 15.30       | 0.14  | 3 | ns        |
| MCHC  | 29.88               | 0.47   | 5 | 30.10           | 0.82   | 6 | 29.90       | 0.00  | 3 | ns        |
| Differential counts                                       |                     |        |   |                 |        |   |             |       |   |           |
| neutrophils   | 21.00               | 12.43  | 5 | 16.17           | 6.43   | 6 | 9.00        | 4.24  | 3 | ns        |
| lymphs  | 76.80               | 14.45  | 5 | 77.83           | 8.13   | 6 | 86.50       | 4.95  | 3 | ns        |
| eosinophils   | 1.80                | 2.39   | 5 | 6.00            | 4.38   | 6 | 4.50        | 0.71  | 3 | ns        |
| monocytes   | 2.00                |        | 1 |                 |        |   |             |       |   |           |
| Chemistry   |                     |        |   |                 |        |   |             |       |   |           |
| albumin   | 1.45                | 0.21   | 2 | 1.30            | 0.14   | 2 | 1.50        |       |   | ns        |
| alk phosphatase   | 165.40              | 97.94  | 5 | 145.00          | 15.39  | 3 |             |       |   | ns        |
| ALT/SGPT  | 227.67              | 220.37 | 3 | 223.33          | 102.76 | 3 |             |       |   | ns        |
| AST/SGOT  | 86.20               | 25.93  | 5 | 191.50          | 27.58  | 2 |             |       |   | * p<0.005 |
| BUN   | 19.33               | 1.97   | 6 | 19.25           | 4.03   | 4 | 23.00       | 5.66  | 3 | ns        |
| creatinine  | 0.20                | 0.00   | 7 | 0.22            | 0.08   | 6 | 0.10        | 0.00  | 4 | & p<0.05  |
| calcium   | 9.13                | 0.14   | 6 | 8.73            | 0.36   | 4 | 8.65        | 0.49  | 3 | ns        |
| CO2   | 20.50               | 2.59   | 6 | 16.75           | 3.50   | 4 | 18.00       | 2.83  | 3 | ns        |
| sodium  | 149.33              | 0.82   | 6 | 148.25          | 1.50   | 4 | 147.50      | 2.12  | 3 | ns        |
| potassium   | 4.35                | 0.36   | 6 | 4.05            | 0.53   | 4 | 4.30        | 0.14  | 3 | ns        |
| chloride  | 114.40              | 2.88   | 5 | 116.75          | 3.40   | 4 | 110.00      | 7.07  | 3 | ns        |
| total protein   | 5.00                | 0.14   | 2 | 4.05            | 0.21   | 2 | 4.70        |       | 2 | ns        |
| Glucose   | 113.43              | 17.64  | 7 | 104.00          | 32.90  | 6 | 61.00       | 22.65 | 4 | ns        |

**Supplementary 3 Table 6. Hematology Results Allogeneic vaccine for Melanoma Model. Data 2 weeks post B16aGal vaccines**

|                            | <b>Allogeneic Melanoma model 2 weeks after B16aGal Vaccine</b> |       |   |                 |      |   |             |      |   |        |
|----------------------------|--|-------|---|-----------------|------|---|-------------|------|---|--------|
|                            | B16aGal vaccine group  |       |   | RRBC Only group |      |   | Naive group |      |   | ANOVA  |
| <b>Hemogram</b>            | mean   | sd    | n | mean            | sd   | n | mean        | sd   | n | p>0.05 |
| WBC                        | 24.8   | 26.1  | 5 | 11.8            | 1.1  | 4 | 26.0        | 8.6  | 4 | ns     |
| RBC                        | 8.2  | 0.9   | 5 | 8.0             | 0.2  | 3 | 8.3         | 1.0  | 4 | ns     |
| Hemoglobin                 | 13.4   | 1.5   | 5 | 13.2            | 0.1  | 3 | 13.6        | 1.3  | 4 | ns     |
| Hematocrit                 | 74.4   | 6.1   | 5 | 71.9            | 0.3  | 3 | 75.3        | 6.7  | 4 | ns     |
| MCV                        | 90.9   | 3.3   | 5 | 90.0            | 1.8  | 3 | 91.5        | 3.4  | 4 | ns     |
| MCH                        | 16.3   | 0.2   | 5 | 16.5            | 0.2  | 3 | 16.5        | 0.6  | 4 | ns     |
| MCHC                       | 18.0   | 0.7   | 5 | 18.3            | 0.2  | 3 | 18.1        | 0.2  | 4 | ns     |
| Platelet - Auto            | 905.6  | 247.7 | 5 | 833.3           | 39.6 | 3 | 966.1       | 78.2 | 4 | ns     |
| <b>Differential Counts</b> |  |       |   |                 |      |   |             |      |   |        |
| Neutrophil                 | 2.4  | 2.6   | 5 | 1.5             | 0.9  | 4 | 2.6         | 1.1  | 4 | ns     |
| Band Neutrophil            | 0.0  | 0.0   | 5 | 0.0             | 0.0  | 4 | 0.0         | 0.0  | 4 | ns     |
| Lymphocyte                 | 20.0   | 21.4  | 5 | 9.7             | 2.0  | 4 | 21.0        | 7.5  | 4 | ns     |
| Monocyte                   | 0.5  | 0.4   | 5 | 0.2             | 0.2  | 4 | 0.3         | 0.2  | 4 | ns     |
| Eosinophil                 | 1.9  | 1.9   | 5 | 0.4             | 0.3  | 4 | 2.1         | 0.5  | 4 | ns     |
| Basophils                  | 0.0  | 0.0   | 5 | 0.0             | 0.0  | 4 | 0.0         | 0.0  | 4 | ns     |
| RDW                        | 20.2   | 1.3   | 5 | 18.7            | 0.7  | 3 | 19.2        | 1.6  | 4 | ns     |
| Nucleated RBC              | 0.0  | 0.0   | 5 | 0.0             | 0.0  | 4 | 0.0         | 0.0  | 4 | ns     |
| <b>Chemistry</b>           |  |       |   |                 |      |   |             |      |   |        |
| BUN                        | 23.8   | 2.6   | 6 | 22.0            | 6.2  | 4 | 24.0        | 4.3  | 5 | ns     |
| Creat                      | 0.1  | 0.1   | 6 | 0.2             | 0.0  | 4 | 0.2         | 0.0  | 5 | ns     |
| Glucose                    | 106.8  | 28.4  | 6 | 111.3           | 21.0 | 4 | 129.6       | 26.7 | 5 | ns     |
| Total Protein              | 5.2  | 0.1   | 6 | 5.5             | 0.3  | 4 | 5.0         | 0.5  | 5 | ns     |
| Albumin                    | 3.5  | 0.3   | 6 | 3.8             | 0.3  | 4 | 3.3         | 0.3  | 5 | ns     |
| Alk Phos                   | 95.2   | 13.1  | 6 | 96.3            | 17.9 | 4 | 107.0       | 29.0 | 5 | ns     |
| ALT                        | 69.8   | 29.4  | 6 | 34.0            | 3.9  | 4 | 62.6        | 55.2 | 5 | ns     |
| Calcium                    | 10.1   | 0.1   | 6 | 10.6            | 0.2  | 4 | 10.1        | 0.3  | 5 | ns     |





**Supplementary 4 Table 3. Histopathology Results. Incidence of microscopic lesions in the Allogeneic vaccine for Breast Cancer Model**

|   | <b>Histopathology after 1 week of allogeneic EMT-6 cancer vaccine</b> |              |               |               |             |              |
|---|---|--------------|---------------|---------------|-------------|--------------|
|   | <b>Lungs</b>  | <b>Liver</b> | <b>Spleen</b> | <b>Kidney</b> | <b>Skin</b> | <b>Heart</b> |
| <b>EMT-6 Vaccine group</b>  | 2/4   | 0/4          | 0/4           | 1/4           | 0/4         | 0/4          |
| <b>RRBC only group</b>  | 2/4   | 0/4          | 0/4           | 1/4           | 0/4         | 0/4          |
| <b>Naïve group</b>  | 2/4   | 0/4          | 0/4           | 1/4           | 0/4         | 0/4          |
| Lungs: mild inflammation. Cuffing pneumonia<br>Kidneys: Mild multifocal accumulations of lymphocytes surrounding larger arterioles. |   |              |               |               |             |              |

**Supplementary 4 Table 4. Histopathology Results. Incidence of microscopic lesions in the Allogeneic vaccine for Breast Cancer Model**

|  | <b>Histopathology after 6 months of allogeneic EMT-6 cancer vaccine</b> |              |               |               |             |
|--|---|--------------|---------------|---------------|-------------|
|  | <b>Lungs</b>  | <b>Liver</b> | <b>Spleen</b> | <b>Kidney</b> | <b>Skin</b> |
| <b>EMT-6 Vaccine group</b>   | 8/8   | 5/8          | 0/8           | 7/8           | 0/8         |
| <b>RRBC only group</b>   | 4/6   | 3/6          | 0/6           | 3/6           | 1/6         |
| <b>Naïve group</b>   | 2/3   | 2/3          | 0/3           | 2/3           | 0/3         |
| Lungs: Moderate multifocal nonsuppurative peribronchiolar and perivascular cuffs. Moderate diffuse atelectasis and congestion<br>Liver: Mild periportal accumulations of mononuclear cells.<br>Kidneys: Mild to moderate multifocal nonsuppurative interstitial nephritis<br>Skin: A single lymphoid aggregate is present in dermis. |   |              |               |               |             |







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